Applicability of a Biotest Battery Developed for Temperate Regions to Tropical Environments: Implications for Sustainable Wetland Management –

A Case Study of Kilombero Ramsar Site

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Applicability of a Biotest Battery Developed for Temperate Regions to Tropical Environments: Implications for Sustainable Wetland Management – A Case Study of Kilombero Ramsar Site

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Abbreviations

AAT	Acute Algae Test
AGI	Algae Growth Inhibition Test
AMPA	Amino-methylphosphonic acid
ANOVA	Analysis of Variance
BCA	Bacteria Contact Assay
Chita Ns	Chita National Services farms
DCMU	3-(3, 4-dichlorophenyl)-1,1-dimethylurea
DF	Delayed fluorescence
DIN	Deutsches Institute für Normung (Germany Industrial Standards)
DMSO	Dimethyl sulfoxide
DSM	*Arthrobacter globiformis* culture medium
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
DW	dry weight
EC	Electrical conductivity
EC_{50}	Half maximal effective concentration
EIA	Environmental Impact Assessments
EPSPS	5-Enylpyruvylshikimate-3-phosphate synthase
FAO	Food and Agriculture Organization of the United Nations
FRB	Fuzzy Rule Base
GBA	Gesellschaft für Bioanalytik mbH
GDP	Gross domestic product
GPS	G*lobal Positioning System*
ha	Hectares
HAW	Hamburg University of Applied Sciences
HPLC-MS	High Performance Liquid Chromatography - Mass Spectrometry
IC_{50}	Half maximal inhibitory concentration
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
IP	Idete agricultural prison
ISO	International Organization for Standardization

ISRIC	International Soil Reference and Information Centre
Koc	Soil organic carbon-water partitioning coefficient
KP	Kiberege agricultural prison
KPL	Kilombero Plantations Ltd
KSC	Kilombero sugarcane plantations
KVRS	Kilombero Valley Ramsar Site
LBT	Luminescence Bacteria Test
LC_{50}	Lethal concentration (50%)
MBS	Mbingu sisters farms
MOP	3-(N-morpholino) propanesulfonic acid
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NEMC	National Environmental Management Council of Tanzania
OD	Optic density
OECD	Organization for Economic Co-operation and Development
PBS	Phosphate-buffered saline
PAM	Pulse Amplitude Modulation
PCA	Principal Component Analysis
PF	Prompt fluorescence
PS II	Photosystem II
rpm	Revolution per minute
RuBP	Ribulose biphosphate
SAG	Sammlung von Algenkulturen der Universität Göttingen
SAGCOT	Southern Agricultural Corridor of Tanzania
SRM	Standard Reference Material
TAC	Tanganyika Agricultural Research Centre
TBS	Tanzanian Bureau of Standards
Teak Co.	Kilombero valley teak Company
TPRI	Tropical Research Institute of Tanzania
UNESCO	The United Nations Educational, Scientific and Cultural Organization
URT	United Republic of Tanzania
XRFA	X-Ray Fluorescence Analysis

YT	Yeast Test
-ve	Negative

SUMMARY

The increasing rate at which chemicals are released into the environment in Africa over the past two to three decades has raised a concern about the immediate ecological impacts and associated long-term environmental risks. Toxicity tests, which measure responses of organisms to various contaminants, have the capacity to indicate ecological impacts of chemicals that are released into the environment, and are therefore used in risk assessment studies. Intensive eco-toxicological studies in the developed world have led to establishment of standardized biotest procedures in order to assure reproducible and reliable results for risk assessment and for environmental regulations. Although there are a lot of data and publications on water and sediment quality for temperate countries using bioassays, tropical ecological risk assessment data using bioassays are scarce.

The focus of this study was to apply biotests developed in temperate countries on tropical samples and evaluate their suitability for assessing pesticide contamination in soil, water, and sediment from Kilombero Valley Ramsar Site, in Tanzania. A total number of 143 dry and rainy season samples (58 water, 68 sediments and 17 soils) was collected in eight plantations (sugarcane, rice and teak). Toxicities were assessed by a battery of standardized tests comprising the algae growth inhibition test with the freshwater green algae, *Pseudokirchneriella subcapitata,* the luminescence bacteria test with *Vibrio fischeri*, and the bacterial sediment contact test with *Arthrobacter globiformis*. Responses of test organisms to sample matrices were categorized and classified into toxicity classes according to a fuzzy rule based-expert system. Suitability of using the *P. subcapitata* bioassay for assessing pesticide contamination in tropical agronomic systems based on the results of this study was evaluated. Chemical, geochemical and eco-toxicological analyses were combined in a weight of evidence approach to provide environmental risk information for this Ramsar wetland (wetland of national and international importance according to the Ramsar Convention of 1971). Interviews with plantation managers were conducted in order to get an overview over the application of pesticides in the area. Together with the distribution of measured pesticides, an evaluation of potential human risk has been carried out.

Toxicity responses of the three bioassays differed to a considerable extent depending on the sample matrices and season. Dry season samples (soil and sediments) resulted in higher mean inhibition of *A. globiformis* (25%-31%) than rainy season sediment samples (5%). Neither *P.*

subcapitata nor *V. fischeri* showed a significant difference between rainy and dry season samples (water and sediments) even though there was a trend towards higher inhibition during dry season. Growth of *P. subcapitata* was stimulated (to an average of 69%) by all dry season soil samples while *V. fischeri* and *A. globiformis* were inhibited by the same samples at mean values of 34% and 25%, respectively. The observation that toxicities tended to be higher in dry than in rainy season sediment samples in the three bioassays indicate the presence of bioavailable inhibiting contaminants, which are deposited in the banks of streams or rivers when the flow velocity is slow. Low toxicities of rain season samples probably indicate a lower concentration of pesticides/contaminants due to flooding and increased surface runoff. A few dry season sediment samples collected from the outlets streams that receive drains from the rice paddies, as well as those collected adjacent to pesticide mixing /sprayer filling point, showed elevated toxic responses in three bioassays. However, there were no a direct relationship between such samples and the detected pesticides residuals, suggesting that the toxicity was caused by other contaminants. Fuzzy rule based classification of toxicity responses of the three bioassays showed that 73% of samples posed little or no toxic potential risk, while 25% were identified to pose critical risk and only 1% posed elevated critical risk.

The algae growth inhibition test showed stimulation in 131 samples out of 143 rainy and dry season samples (sediments, soil and water). Laboratory experiments to evaluate the source of stimulation in the algae test revealed that nutrients such as organic carbon and Vitamin B in the test medium/matrices could stimulate *P. subcapitata* growth rate. Kilombero samples, however, had relatively low C/N ratios (according to FAO-classification), varying from 10 to 20 for poorly drained soils, suggesting that there is high decomposition of organic matter (enhanced by tropical climatic conditions) and organic humus is transported from the highlands to these lowland flood plain wetlands, thus enriching its organic nutrients.

Experiments were carried out to assess whether the blockage of electron transport system by photosystem II inhibitors (specific herbicides) could have caused the stimulation in the *P. subcapitata* growth inhibition test. It was revealed that: as opposed to chemicals with different mode of action, the presence of PS-II inhibitors in the test medium stimulated the prompt fluorescence (PF) and inhibited the delayed fluorescence (DF), a pattern that is caused by back reactions when the electron transport system is blocked. Although pesticide residuals such as diuron, glyphosate, AMPA, propoxur, atrazine, 2-hydroxy-atrazin, metribuzin, chlorpyrifos,

desmethyldiuron, monuron, hexazinone and ametryin were detected in water, sediment and soil samples, the DF and PF patterns were not detected in the environmental samples as they were with single substance tests. Despite the potential to induce fluorescence, the presence of PSII inhibitors in the samples could thus not be responsible for stimulation in the 72-hour growth inhibition test.

In conclusion, *V. fischeri* and *A. globiformis* bioassays can be used in tropical climates to identify contaminated hotspots. However, the chronic *P. subcapitata* bioassay could not provide enough information on the toxicity of sample matrices because of the observed stimulations. Thus, there might be a potential to underestimate the toxicity of samples collected from agricultural fields, especially for sediment and soil samples, which are rich in nutrients and are contaminated with low concentration of pesticides. Supplementing the 72-hour algae test with a short bioassay, detecting photosystem II inhibitors, could provide additional, useful information on the toxicity of herbicides in environmental samples.

Another supplementary test with the yeast *Saccharomyces cerevisiae* showed toxic responses in the same magnitude as in the bacterial bioassays when applied to the Kilombero samples. While promising to become a meaningful addition to the biotest battery to indicate fungicides, this test needs optimization due to unexplained reactions when exposed to organic pesticides.

The risks of pesticides in the ecosystem depend on the characteristics of a chemical and on the level and pattern of exposure. Unsafe handling of pesticides in the Kilombero valley, which were revealed during field surveys are: pesticide spillage at retail marketing places, spillage during filling up the sprayers, cleaning of the spraying equipment in open waters, on-farm disposal of the pesticide containers and mixing pesticides close to streams, wetlands and ditches. These water bodies surrounding the surveyed plantations have been used by the adjacent households as a source of water for domestic use. Therefore, continued use and unsafe handling of pesticides in these wetlands pose a great risk to human health, wildlife and aquatic organisms. Ecotoxicology using simple, fast and cost effective bioassays, which are novel to Tanzanian setting, might help to provide strategies for reducing the risk from agrochemicals in the Tanzanian wetland ecosystems.

Zusammenfassung

Die zunehmende Menge an Chemikalien, die während der letzten zwei bis drei Jahrzehnte in Afrika in die Umwelt eingetragen wurde, ist wegen der unmittelbaren ökologischen Folgen und wegen der langfristigen Risiken besorgniserregend. Toxizitätsstudien, die die Reaktionen von Organismen auf verschiedene Kontaminanten messen, können potenzielle ökologische Folgen einer Chemikalienapplikation anzeigen und werden daher in Risikobewertungen eingesetzt. Intensive ökotoxikologische Studien in der entwickelten Welt haben zur Etablierung standardisierter Biotestverfahren geführt, um reproduzierbare und zuverlässige Daten für die Bewertung von Risiken und zur Entwicklung von Umweltregulatorien zu erhalten. Während in gemäßigten Regionen viele Daten und Publikationen zur Wasser und Sedimentqualität mit Hilfe von Biotests erhoben werden, werden für ökologische Risikobewertungen in den Tropen selten ökotoxikologische Testmethoden eingesetzt.

In dieser Studie wurden Sediment-, Boden- und Wasserproben aus Kilombero Valley Ramsar Site, Tansania, mit Hilfe verschiedener Bioassays, die in gemäßigten Zonen entwickelt wurden, getestet. Dabei sollte untersucht werden, inwiefern sie geeignet sind, um Pestizid-Kontaminationen in den verschiedenen Matrizes anzuzeigen. Insgesamt wurden 143 Proben während der Trocken- und der Regenzeit in 8 Plantagen (Zuckerrohr, Reis und Teakholz) genommen (58 Wasser-, 68 Sediment- und 17 Bodenproben). Toxizitäten wurden mit folgenden standardisierten Biotests untersucht: Mit dem Algenwachstumshemmtest mit der Süßwasseralge *Pseudokirchneriella subcapitata*, mit dem Leuchtbakterientest mit *Vibrio fischeri* und mit dem Bakterienkontakttest mit *Arthrobacter globiformis*. Mit Hilfe eines Expertensystems auf der Grundlage einer Fuzzy-Regelbasis wurden die jeweiligen Hemmungen der Testorganismen kategorisiert und Toxizitätsklassen zugeordnet. Die Eignung des *P. subcapitata* Tests zur Anzeige von Pestizidbelastungen tropischer Umweltproben wurde auf der Basis dieser Daten eingeschätzt. Chemische, geochemische und ökotoxikologische Daten wurden gemeinsam betrachtet. Aus der daraus resultierenden Beweislast (“weight of evidence“ Ansatz) wurden Informationen zum Umweltrisiko für dieses Ramsar-Feuchtgebiet (Feuchtgebiet von nationaler und internationaler Bedeutung entsprechend der Ramsar Convention 1971) abgeleitet. In diesem Rahmen wurden auch Interviews mit den Managern der Plantagen durchgeführt, um einen Überblick über die Anwendung von Pestiziden im Untersuchungsgebiet zu erhalten. Diese Informationen zusammen mit der Verteilung der gemessenen Pestizidkonzentrationen ermöglichten die Einschätzung eines möglichen Risikos für die menschliche Gesundheit.

Die Reaktionen der 3 Biotests unterschieden sich teilweise erheblich in Abhängigkeit der Probenmatrix und der Jahreszeit, in der die Proben genommen wurden. Sediment- und Bodenproben, die während der Trockenzeit genommen worden waren, zeigten eine höhere mittlere Hemmung von *A. globiformis* (25 bis 31 %) als die aus der Regenzeit (5%). Weder bei *P. subcapitata* noch bei *V. fischeri* zeigten sich dagegen signifikante Unterschiede zwischen den Jahreszeiten bei Exposition gegenüber den Sediment- und Wasserproben, auch wenn ein Trend in

Richtung höherer Toxizität in der Trockenzeit erkennbar war. Das Wachstum von *P. subcapitata* im Test wurde durch alle Bodenproben der Trockenzeit stimuliert (Mittelwert 69 %), während *V. fischeri* und *A. globiformis* durch die gleichen Proben zu 34 % bzw. 25 % gehemmt wurden. Die tendenziell höhere Toxizität in der Trockenzeit im Vergleich zur Regenzeit deutet darauf hin, dass Schadstoffe vorhanden und verfügbar sind, die sich am Ufer von Bächen und Flüssen ablagern, wenn die Strömungsgeschwindigkeit gering ist. Die geringen Hemmungen durch die während der Regenzeit genommenen Proben kommen vermutlich durch einen erhöhten Abtrag der Sedimentoberflächen aufgrund von Überschwemmungen zustande. Einige Proben, die in der Trockenzeit von den Mündungen der Entwässerungskanäle der Reisfelder bzw. nahe der Auffüllstation für die Pestizid-Anmischung genommen wurden, zeigten erhöhte Toxizitäten in allen drei Biotests. Es zeigte sich jedoch kein direkter Zusammenhang zwischen den ökotoxikologischen Daten und den Pestizidmessungen. Dies lässt vermuten, dass andere Schadstoffe, die nicht analysiert worden sind, die toxische Wirkung hervorgerufen haben. Eine Klassifizierung durch das Fuzzy-Expertensystem resultierte in 73 % aller Proben, die ein geringes oder kein potenzielles Risiko anzeigten, während das Risiko in 25 % Proben kritisch und nur in einem Prozent erhöht kritisch war.

Der Algenwachstumshemmtest war in 131 von 143 Proben stimuliert (Sedimente, Böden, Wasser; Trocken- und Regenzeit). In Laborexperimenten konnte gezeigt werden, dass zusätzliche Nährstoffe wie organischer Kohlenstoff und Vitamin B im Testmedium die Wachstumsrate von *P. subcapitata* stimulierten. Die Kilombero-Proben hatten jedoch geringe C/N Verhältnisse (nach FAO-Klassifizierung) zwischen 10 und 20 für schlecht drainierte Böden. Dies lässt auf einen hohen Abbau des organischen Materials (verstärkt durch tropische klimatische Bedingungen) schließen, sowie auf einen Transport von Humusstoffen vom Hochland in die tiefer gelegenen Feuchtgebiete in der Flussaue, wodurch die organischen Nährstoffe angereichert werden.

Weitere Experimente sollten zeigen, ob die Blockade des Elektronentransports durch Inhibitoren des Photosystems II (bestimmte Herbizide) die Stimulation im Algenwachstumshemmtest hervorgerufen haben könnten. Es zeigte sich, dass - im Gegensatz zu Schadstoffen mit einem anderen Wirkmodus – PSII-Inhibitoren im Testmedium die prompte Fluoreszenz (PF) der Algen stimulierten, während sie die verzögerte Fluoreszenz (DF) hemmten. Dieses Muster wird von rückwärts gerichteten Reaktionen hervorgerufen, wenn das Elektronentransportsystem blockiert ist. Im Gegensatz zu den Einzelsubstanztests im Labor wurden diese Muster in den Umweltproben nicht gefunden, obwohl Pestizidrückstände wie Diuron, Glyphosat, AMPA, Propoxur, Atrazin, 2-Hydroxy-Atrazin, Metribuzin, Chlorpyrifos, Desmethyldiuron, Monuron, Hexazinone and Ametryin in Wasser-, Sediment- und Bodenproben gefunden wurden. Trotz des Potenzials der PSII-Inhibitoren, die prompte Fluoreszenz zu erhöhen, konnte die Stimulation im Algentest dadurch somit nicht erklärt werden.

Schlussfolgernd lässt sich sagen, dass die Biotests mit *V. fischeri* und *A. globiformis* genutzt werden können, um Belastungshotspots in Proben aus tropischen Klimazonen zu identifizieren. Der Informationsgehalt des Algenwachstumshemmtests mit *P. subcapitata* dagegen war aufgrund der fast durchgehend hohen Stimulation gering. Durch die Stimulation könnte die Toxizität der Proben

aus den landwirtschaftlichen Feldern unterschätzt werden, insbesondere in solchen Sediment- und Bodenproben, die nährstoffreich und nur gering belastet sind. Eine Ergänzung des standardisierten Algentests, der über 72 Stunden läuft, mit einem kurzen Bioassay, der Photosystem II Inhibitoren anzeigt, könnte zusätzliche nützliche Informationen über die Toxizität von Herbiziden in Umweltproben liefern.

Ein weiterer ergänzender Test mit *Saccharomyces cerevisiae* zeigte toxische Hemmungen bei den Kilombero-Proben in der gleichen Größenordnung wie die bakteriellen Biotests. Während dieser Test das Potenzial hat, Fungizide in den Proben anzuzeigen, sollte er noch weiter untersucht und optimiert werden, da er nicht-erklärbare Reaktionen bei der Exposition gegenüber organischen Pestiziden zeigte.

Die Risiken durch Pestizide im Ökosystem hängen von den Charakteristika der Verbindungen ab sowie von dem Ausmaß und der Art der Exposition. Felduntersuchungen zeigten, dass mit Pestiziden im Kilombero-Tal fahrlässig umgegangen wird, z.B. in Form von Verschütten von Pestiziden beim Einzelverkauf und beim Auffüllen der Sprayer, durch das Säubern der Sprühgeräte in Gewässern, durch das Wegwerfen von Pestizid-Containern auf Farmland und durch das Anmixen von Pestiziden in direkter Nähe von Flüssen, Feuchtgebieten und Gräben. Die Gewässer in der Umgebung der untersuchten Plantagen werden jedoch von den angrenzenden Haushalten als Wasserquelle genutzt. Der fortgesetzte Gebrauch und die fahrlässige Handhabung von Pestiziden in diesen Feuchtgebieten birgt ein großes Risikopotenzial für die menschliche Gesundheit, für die Flora und Fauna und für aquatische Organismen. Ökotoxikologische Untersuchungen mit einfachen, schnellen und kosteffizienten Testmethoden, die neu für Tansania sind, könnten helfen, um Strategien zu entwickeln, das Risiko durch Agrochemikalien in tansanianischen Feuchtgebieten zu reduzieren.

CHAPTER ONE: INTRODUCTION

1.1 General introduction

Over the past three to four decades the amount of chemicals released into the environment have increased worldwide. This has raised many concerns especially on the long-term impacts of such toxic and non-toxic chemicals to the surrounding ecological communities particularly on the apparent damage and other associated environmental risks. From this perspective, the role of standardized test protocols in eco-toxicology has been intensified in order to enable detection, monitoring and control of chemical pollution by regulatory authorities and industries (Allan *et al*., 2006). Toxicity tests measure responses of living organisms to various contaminants and thus can be used by authorities to regulate the discharge rate of chemicals to the surrounding environment in order to reduce ecological effects of toxicants to organisms. Development of controlled laboratory toxicity tests using invertebrates and micro organismic species is much advanced, because conducting toxicity tests using vertebrates or higher animal species for ecological risk assessment researches is subject to animal ethics and prevailing laws of a country or region (Gandrass & Salomons, 2001). Estimates of environmental damage are extrapolated from the laboratory response of specific test organisms´ level of biological organization to entire natural systems (Gurney *et al*., 1996; Kooijman *et al*., 1989). However, the challenges of eco-toxicology research remain on the understanding of the complexity of ecological interrelationships (Calow *et al*., 1997; McCauley *et al*., 1990). The crucial motivation in ecological risk assessments is the necessity to investigate which processes within test organisms are responsible for changes in life-history traits, and how these processes are affected by toxic stress (Calow & Sibly, 1990; Kooijman, 1986; McCauley *et al*., 1990; Sibly, 1996). In recognition of the magnitude of time and expenses required to perform life-history experiments for a broad set of organisms: rapid, acute and chronic toxicity tests methods that use a range of indicator species have been developed and intensified in the temperate countries for the past two to three decades. Such methods use living cells, tissues, enzymes, physiological responses, organism or communities in a defined type and amount to assess exposure-related effects of chemicals (Repetto, 2013). Biotests utilize the capacity of specific organisms to respond to exposure of contaminants under standardized conditions by change in their vital functions. Eco-toxicological testing using sets of species and different endpoint measurements provides an insight about the effect in general without pinpointing the potential

source of the substances involved. Therefore, bioassays can be used for various purposes, such as bio-monitoring (Allan *et al*., 2006) and early warning systems (Power & Boumphrey, 2004), regulatory purposes (Power & Boumphrey, 2004), effect screening (Bosveld & Berg, 1994), research and teaching, and hazard/risk assessment (Joern & Hoagland, 1996; Lyne *et al*., 1992). Hence, field studies combined with analytical assessments are essential to verify results from the laboratory biotests, especially when sub-cellular test systems or surrogate organisms are applied. Chemical analysis detects and keeps records of parental substances or toxicants and their metabolites, but does not provide information on the effect of the pollutants to organisms or about synergetic and antagonistic factors. Although the biotest methods are applied under standardized and controlled non-field conditions, the measured biological end-points can be extrapolated to biota potentially affected by environmental exposures. A combination of different test methods referred to as a battery of biotests, with different test organisms at different trophic levels which have different sensitivities to contaminants, are therefore preferred (Allan *et al*., 2006). Interpretations of results from a battery of biotests, as an integrative assessment, provide an opportunity to identify and prioritize hotspot and actual bioavailability of contaminants, thus providing a reliable, valid and reproducible tool in the determination of ecological risk assessment (Johnson *et al*., 2005; Wolska *et al*., 2010; Wolska *et al*., 2007). According to Urban and Cook (1986), ecological risk assessment refers to estimating the likelihood or probability that adverse effects (such as mortality to a single species of organisms, or reductions in populations of non-target organisms due to acute, chronic and reproductive effects, or disruption in community and ecosystem level functions) have occurred, are occurring or will occur. Risk assessment is scientifically based on evaluation of hazard and exposure data to determine a presumption of risk (Chapman *et al*., 1998) which deliver the outcomes in a simple, non-technical language for both scientific and general society to comprehend and manage the risk. Therefore, it includes the processing of choosing a course of action that reduces the potential impact (Levin *et al*., 1989). It also involves scientific judgment, political, social and economic consideration in the decision-making process (Urban & Cook, 1986). Legislated toxicity test and procedures used in the regulatory community necessarily follow the development of methods in the research community whereby ecological consequences of environmental contamination are determined by a complex interplay of cause and effect.

Moreover, organisms are affected by multiple environmental stresses; thus sensitivity, ability to survive or adapt to such altered community structure and functions depend on individual organisms and the threshold value of the contaminants. Each level of biological organisms has its

own characteristics, structures and behaviors (Dale & Beyeler, 2001). Since chemicals have unique effects at different levels of complexity and time-space scale, then effects of certain contaminants also depend on what is measured, where it is measured, and how long it is measured (Schaeffer *et al*., 1988). Eco-toxicological test systems can address problems that might occur at large and in complex systems, spot the effects of bio-available contaminants including their potential interactions and provide an insight of the effect of low levels of contaminants, which cannot be detected by analytical means. Although intensive eco-toxicological studies have been conducted in the developed world, where the standardized test protocols have been developed, less have been done in third world countries, most of which are in the tropical region. Consequently, there is scarcity of tropical-Africa eco-toxicological data using tropical model organisms in risk assessment studies (Lacher & Goldstein, 1997; Peters e*t al* 1997; Utembe & Gulamian, 2015) a problem that accelerates the use of temperate risk assessment models to estimate ecological risks for tropical areas. In nutshell, this study was conducted in order to evaluate the suitability of using temperate biotest batteries and their model organisms for assessing pesticide contamination in tropical agrarian systems. Therefore, a set of three standardized bioassays using freshwater green algae and bacteria, and two non-standardized acute bioassays using freshwater green algae and fungus were tested. The results of seasonal toxicities of sediment, soil and water samples from Kilombero Valley Ramsar site, Tanzania, were evaluated and critically discussed based on the suitability of using such temperate biotest batteries in risk assessment studies of tropical agronomic systems.

1.2 Background

Wetland ecosystems are relatively fertile, rich in biodiversity and can support a number of economic activities such as farming, fishing and grazing (Jos *et al*., 2010). For developing countries, like Tanzania, wetlands are beneficial for food crop production and other livelihood activities particularly to communities surrounding them (McCartney & Van Koppen, 2004a; McCartney & Houghton-Carr, 2009; Turyahabwe *et al*., 2013). It is notable that Tanzania is rich in river basins and wetland resources with an estimated 10% of the land surface covered by significant freshwater wetlands (MNRT, 2003). Although wetland agriculture has historically existed for long time in rural majority, a current trend of encroachment and expansion of cultivation areas has been witnessed in both protected and unprotected wetlands. For the past two to three years, Tanzanian government has politically solicited large-scale agribusiness investments and projects in the fertile wetland ecosystems, partly in order to combat poverty and increase food security during drought seasons

(SAGCOT, 2011). However, these initiatives seem to ignore the potential alterations of ecological character of the wetlands. Wetland agriculture if not sustained, might modify the flow and nature of the whole river basin (Madulu, 2005). Although the socio-economic values of wetlands in Tanzania are comprehensively documented (FAO, 2003; Kangalawe & Liwenga, 2005; Madulu, 2005; 2003; Mombo *et al*., 2011), there is no sufficient data on the extent of ecological destruction of the wetlands by human activities and on the sustainability of intensified agricultural projects in the wetlands. For example, under KILIMO KWANZA political declaration of 2009, the Tanzanian government has earmarked the country´s southern highlands, where Kilombero Ramsar Site is located, under SAGCOT project. This project entails large-scale agricultural investments and long-term land lease to private investors for purposes of mechanized agriculture. It is anticipated that full implementation of these massive agricultural projects will put considerable stress on river catchments and wetland ecosystems.

Contemporary issues for ecological destruction and unsustainable use of wetland ecosystems for agriculture are the trend of unrestricted pesticide use, lack of proper monitoring of the pesticide utilization; and insufficient knowledge of local people on the impact of the agrochemicals to human health and environment. Although intensive eco-toxicological studies of pesticides coupled with analytical assessment surrounding agricultural fields are well documented for temperate countries (Beitz *et al*., 1994; Giesy *et al*., 2000; Kendall, 1992; Solomon *et al*., 1996; Steen *et al*., 1999), only quantitative studies of pesticides detected in the environment are reported in Tanzania (Kishimba *et al*., 2004; Mdegela *et al*., 2009; Mmochi & SAID Mberek, 1998; Mwevura *et al*., 2002) and elsewhere in tropical countries (Kannan *et al.*, 1995; Laabs *et al*., 2002b; Thapinta & Hudak, 2000). If the available analytical information is combined with eco-toxicological data, then comprehensive risk assessment and monitoring programs of the impacts of agricultural effluents into aquatic ecosystems of Tanzania might be developed. Well established and tested bioassays for Tanzanian case and other tropical countries, might help to reduce high costs incurred for analytical assessments, thus becoming a useful tool for monitoring ecosystem damage in the future. This study applied a battery of standardized biotests developed in the temperate countries to assess the eco-toxicity of water, soil and sediment samples collected from Kilombero Valley Ramsar site, as an illustrative study for tropical agronomic systems.

1.3 Literature review

1.3.1 The use of biotests for risk assessment

Eco-toxicology is an interdisciplinary environmental science which deals with the interactions between environmental chemicals and biota, thereby focusing on adverse effects of such chemicals at different levels of biological organizations (Fent, 2003). This field encompasses three main disciplines, which are toxicology, applied ecology and environmental chemistry. Ecological surveys use biomarkers to verify the bioavailability of contaminants (Fent, 2004) and establish potential ecological risk of contaminants in the biota (Bucheli & Fent, 1995). However, ecological extrapolation of laboratory bioassays results to estimate possible effects on the ecosystems, is limited by environmental factors such as multitude of chemicals in ecosystems, species diversity, biological functions and structure (Fent, 2004). Chemical and physical analyses of environmental samples can inform about the amount of single contaminants but are not able to detect all dangerous substances present in such samples (Wadhia & Thompson, 2007).

A number of both acute and chronic toxicity bioassays have been developed and standardized in industrialized countries, such as Canada, USA and Europe, to assess the eco-toxicological impact of contaminants on the aquatic ecosystems (Farré & Barceló, 2003; Wadhia & Thompson, 2007) and are used by regulatory authorities (Wells *et al*., 2001). Application of biotests using different organisms can indicate general stress of complex mixtures of toxicants, and provide information about potential hazard to a preferred ecosystem (Fochtman *et al*., 2000). A battery of biotests provides a wide range of parallel information about ecosystem contamination for further assessment procedures (Mankiewicz-Boczek *et al*., 2008). Species used in eco-toxicological studies have different sensitivities to different contaminants, such as heavy metals (Sauvant *et al*., 1997), organic hydrocarbons and pesticides (Frampton *et al*., 2006; Maltby *et al*., 2005). Therefore, no single species or biotest methods can satisfy the need to assess the quality of the aquatic ecosystem resulting from mixture of pollutants and synergetic or antagonistic effects (Benfenati *et al*., 2003). Application of a battery of bioassays using species from different trophic levels increases both chances of toxicant identification (Wadhia & Thompson, 2007) and the significance for hazard assessment of aquatic ecosystems (Cascorbi *et al*., 1993; Fochtman *et al*., 2000; Manusadžianas *et al*., 2003; Pessala *et al*., 2004). The joint toxicity of chemicals sharing the same mode of action can be predicted by a concentration addition (C A) model (Altenburger *et al*., 2000; Faust *et al*., 2001; Junghans *et al*., 2003; Silva *et al*., 2002; Syberg *et al*., 2008). For instance, Knauer *et al*., (2010) and

Knauert *et al*., (2008; 2009) have revealed the concept of additivity of toxic impact for photosystem II inhibitors and their effect on photosynthesis process of phytoplankton in the aquatic environment. Moreover, chronic tests are argued to be more ecologically relevant than acute tests because they can predict concentrations which affect the growth rate or reproduction of a certain organisms (van Wijngaarden *et al*., 2005). In addition, prediction of sub-lethal toxicant concentrations that can harm an ecosystem is not possible by acute test (Roux *et al*., 1993). Therefore, in this study, both chronic and acute tests were performed using single species in order to estimate the magnitude of environmental harm resulted from toxic chemicals.

1.3.2 Influence of climatic dynamics on ecotoxicology

Influence of climatic factors on sensitivities of species are assessed by comparing responses of organisms from different climatic regions to conditions that denote optimum health (Lewis Jr, 1987). Some studies indicated that the toxicity of chemicals to aquatic biota increases with increasing temperature (Cowgill *et al*., 1985; Heugens *et al*., 2006; Howe *et al*., 1994; Magallona, 1994; Mayasich *et al*., 1986; Racke *et al*., 1997). Tropical species are potentially more sensitive to heavy metals, pesticides such as chlorpyrifos, un-ionized ammonia and phenols contamination than their temperate counterparts (Kwok *et al*., 2007). Higher temperatures decrease the solubility of dissolved oxygen (Viswanathan & Krishnamurti, 1989) and therefore enhance the biochemical detoxication and elimination of some chemicals in the exposed organisms (Racke, 2003). Due to varying climatic patterns, eco-toxicological tests are developed to suit the natural environment of test species and the expected endpoints. Short-term toxicity assays based on the inhibition of enzymes or metabolic activities or physiological parameters of microorganisms and micro-invertebrates such as fungi, bacteria, algae and protozoa are of increasing importance owing to their simplicity, rapidity, cost-effectiveness and reproducibility (Cascorbi *et al*., 1993; Papaefthimiou *et al*., 2004; Ribeiro *et al*., 2000). Species in the high organization levels such as mammals are not often involved in toxicological studies as compared to microorganisms (Cabral *et al*., 2003) because of ethical issues (Gabrielson *et al*., 2003), not being widely distributed, complexity, and slow assimilation of toxicant in the body (Wadhia & Thompson, 2007). In this study inhibition of growth rate of freshwater algae and metabolic activities of bacteria and fungus were used as the end-point responses to assess impacts of contaminated environmental samples to test organisms.

1.3.3 Ecological risk assessment of pesticides in tropical countries

The three components of eco-toxicology i.e. ecology, environmental chemistry and toxicology are affected differently by climatic conditions. The methodological frameworks of eco-toxicology tests and their role in environmental regulations are well developed and applied extensively in temperate countries. In such countries, environmental pollution is considered to directly affect human and economic development following certain regional or interstates frameworks and regulation criteria. In the poor, tropical countries, eco-toxicological studies are very limited, and hence environmental regulations rely on observable effects. In Sub-Saharan Africa, for instance, human population is high in wet areas like river basins, delta and other natural wetlands, oases, and irrigated areas for livelihood activities (Gibbs, 1993; Hook, 1993; Sakané *et al*., 2013; Scoones, 1991). Such wet areas have direct or indirect impacts on the socio-economic activities of local communities or the region in general. Wetland ecosystems are ecologically important and sometimes fall under common pool resources, in such a way that stress of human intervention and destruction throughout its life existence cannot be avoided (Gibbs, 1993; McCartney & Houghton-Carr, 2009). Modernization of agricultural practices in tropical countries have led to increased pesticide use over the past decades (Ecobichon, 2001; Karlsson, 2004; Wilson & Tisdell, 2001), although not as high as in the temperate counterparts. In many tropical countries, pesticides are detected in water, sediment and biota of agricultural areas and surrounding waterways (Domagalski & Kuivila, 1993; Li & Zhang, 1999; Nhan *et al*., 1999; Osano *et al*., 2003; Thapinta & Hudak, 2000; Wandiga, 2001; Zhang *et al*., 2012). Aquatic risk assessments in tropical countries often rely on temperate toxicity data (Castillo *et al*., 1997; Karlsson, 2004; Scoones, 1991), nevertheless environmental fate and effects of toxic chemicals such as pesticides are different between climatic regions. A number of studies indicate that the rate of degradation and distribution of pesticides in tropical regions is higher than in temperate regions (Osano *et al*., 2003; Racke, 2003). Therefore, any extrapolations of risk assessment studies entail a restrained circumstantial evidence (Matsumura, 1989), as there might be no consistence of the detected trends in all seasons and environments.

1.3.4 Influence of climatic factors on fate and distribution of pesticides in the environment

Distribution of pesticides in tropical ecosystems is considered to be higher than in their temperate counterparts due to the influence of rainfall, temperature, sunlight and microbial activities (Magallona, 1994; Racke, 2003). Heavy precipitations play a major role in washing off the pesticides

from the treatment sites, accelerating surface run off, increasing dilution of pesticides in aquatic environment and leaching to ground water. A rainy season in the tropics is associated with large flow of surface particles, dissolved nutrients and toxicants into the water bodies thus potentially posing a toxicological risk to aquatic ecosystems. Particles may adsorb toxicants such as pesticides and enhance their lineage through the water column to the sediment (Goldberg, 1989). The transported nutrients into the water bodies may lead to increased algal biomass, which is further enhanced by the tropical climatic conditions. Pesticides and particles run off to nearby water bodies during high rainy season, disperse from the water phase quite easily due to mixing and dilution, thus leading to enhanced uptake by filter-feeders (Goldberg, 1989). For instance, toxicity of a glyphosate-based herbicide (Roundup®) to the cladoceran-*Ceriodaphnia dubia* is increased with increased suspended sediment concentrations (Tsui & Chu, 2003). Nevertheless, surface particle runoff during heavy rains in tropical ecological zones may result into increased turbidity of shallow waters and hence decrease photolysis and disappearance rates of certain pesticides (Lahr *et al*., 2000). Adsorption of pesticide residues to high concentration of suspended matter with low organic content decreases biochemical degradation of pesticides especially in the aqueous matrices, while increasing the bioavailability in solid phases (Chaplain *et al* 2011). The retention of pesticides is affected by their residence time in soils or solid matrices because of diffusion into soil micropores, physical entrapment or degradation (Koskinen *et al*., 2001).

According to Cox & Walker, (1999), the amount of bound pesticide residues is inversely proportional to mineralization due to the reduction of the microorganism activities and increase of contact times between pesticide and the soil components. Degradation of pesticides such as 2,4-D is reduced (with increase in persistence) in areas characterized by elevated temperatures and limited rainfall (Bouseba *et al.,* 2009). Furthermore, Alletto *et al.* (2006) demonstrated that isoproturon mineralization was largely affected by temperature in both surface and subsurface soils but degradation was more sensitive to water content variations than temperature ones. In colder regions, slight increase of temperature in spring and fall and more frequent freeze-thaw cycles due to climate change modify herbicide degradation, increase leaching and can drastically increase the bioavailability of pesticides such as metribuzin (Benoit *et al*., 2007).

Tropical ecosystems are associated with high temperatures between 25-35°C, higher rate of chemicals hydrolysis (Klein, 1989; Lyman *et al*., 1982; Viswanathan & Krishnamurti, 1989) and higher vaporization rate of pesticides (Magallona, 1994) than temperate ecosystems. Viswanathan and Krishnamurti (1989) showed that solubility of organochlorine insecticides in water increased

with ambient temperature, thus leading to greater uptake and toxicity to aquatic biota. High sunlight intensity in the tropics causes direct photolytic effects and hence enhances photodecomposition (Magallona, 1989). Primary productivity is however increased by fluctuations of diurnal pH in the tropics due to higher solar radiation levels than in temperate regions (Lewis Jr, 1987).

Microorganisms play an important role in degradation of pesticides and other chemicals in the environment (Sethunathan, 1989). Warm and moist environment of tropical climates and cultivation practices for a specific cropping system enhance microorganism communities and increase the potential degradation of pesticides (Magallona, 1994; Matsumura, 1989). For example, Racke *et al*. (1997) associated the flooding of rice paddies that causes reduced surface soil layer with a rapid degradation of chlorinated hydrocarbon and pesticides that contain nitro-group by different types of bacteria. Rapid breakdown of DDT, diazinon and lindane in the sediments of flooded tropical rice fields is associated with presence of anaerobic microorganisms (Magallona, 1989; Sethunathan, 1989). In addition, a fast disappearance of lindane is also demonstrated in flooded temperate soils, with degradation rates comparable to those in tropical flooded soils (Racke *et al*., 1997).

1.3.5 Impacts of pesticide usage in agriculture and public health

The increasing use of agrochemicals worldwide has raised a great challenge on the impacts of chemicals like pesticides to non-target organisms and their overall changes in community responses over time (Van den Brink & Braak, 1999). Both environmental and biological monitoring is essential to evaluate the magnitude of the potential environmental and public health risks of agrochemicals. The use of various pesticides worldwide has proved their positive values in public health especially in vectors control to reduce insect-borne diseases (WHO, 1995). In agriculture, pesticides have helped to increase or improve crop yields per piece of land (Cooper & Dobson, 2007), improve food security, boosting per capita income (Wilson & Tisdell, 2001) and increasing GDP for some developing nations that depend on agriculture (Cooper & Dobson, 2007). Despite the published positive results of using pesticides in agriculture and public health; prolonged use, mismanagement or poor handling of pesticides, especially in developing countries (Ecobichon, 2001; Li & Zhang, 1999), has a potency to become deleterious to environment and public health as well (Carvalho, 2006; Eskenazi *et al*., 1999; 2008; Rekha *et al*., 2006). Moreover, toxicity of broad-spectrum pesticides, environmental persistence and bioaccumulation potential of some pesticides such as

organochlorines and their metabolites cause a simultaneous damage to ecosystems and risks to human health (Bouwman *et al*., 2006; Margni *et al*., 2002; Novotny, 1999). Continuous release of pesticides into the environment can be harmful because not the entire applied chemical reaches the target site. Consequently, each active ingredient of pesticides and their metabolites can be transported from the target site or from one compartment of the environment to another. Dissipation of pesticides in the environment is enhanced by adsorption, volatilization, runoff, spray-drift, leaching and crop removal (Müller *et al*., 2007; van der Werf, 1996), as demonstrated in Fig 1.1, thereby polluting and damaging wide range of biota. The benefits of such pesticide use emanate at the cost of their widespread occurrence in the environment, in which array of abiotic and biotic transformations may lead to more toxic and hazardous products (Carvalho, 2006; Giacomazzi & Cochet, 2004; Salvestrini *et al*., 2002).

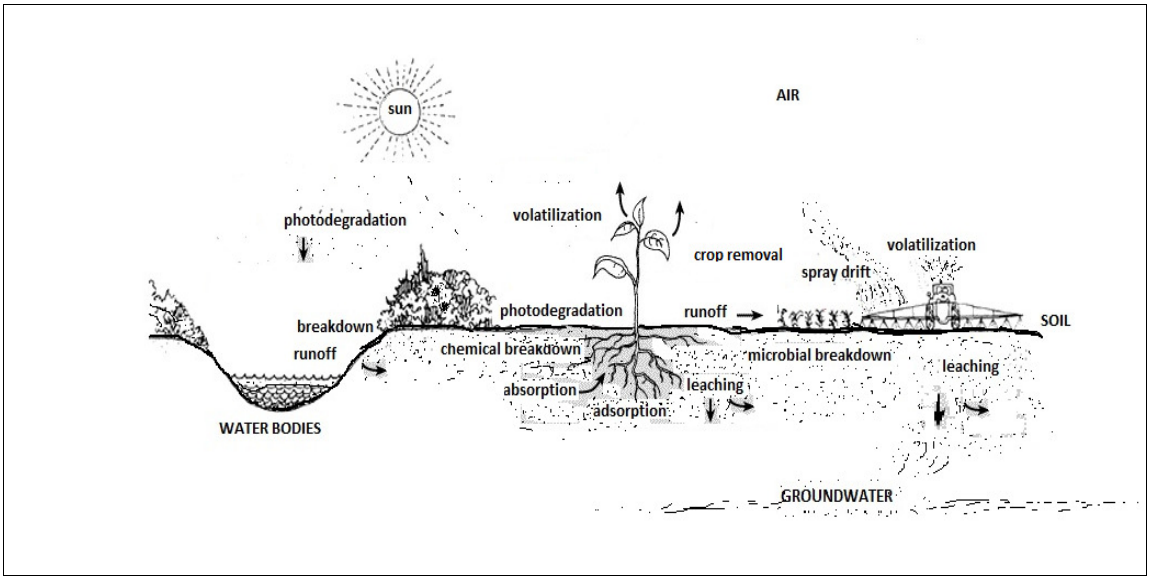


Figure 1.1: A sketch of overview summary of the fate processes of pesticides and other xenobiotic in the environment (modified from: pesticidestewardship.org)

Amount of pesticide adsorbed or bound to soil particles varies with the type of pesticide, soil, moisture, soil pH, and soil texture (Arias-Estévez *et al*., 2008). Pesticides are more strongly adsorbed to soils that are high in clay or organic matter than sandy soils and therefore soil-bound pesticides are less easily taken up by plants or leach through the soil (Gevao *et al*., 2000). Environmental factors (high temperature, low relative humidity, air movement) and soil conditions (texture, moisture, organic matter content) increase pesticide volatilization rate from its target site (Bedos *et al*., 2002).

Moreover, a number of water pollution studies (Kolpin *et al*., 2000; Konda & Pasztor, 2001; Palma *et al*., 2004) reported detection of various pesticides or their metabolites from sediment, surface and ground waters in areas surrounding agricultural fields. It has been forecasted that about 10^9 hectares of natural ecosystems would be converted into agriculture by 2050 due to the agricultural driven global change caused by demand for rapid agricultural expansion, food security and the increasing global population (Tilman *et al*., 2001). Any effort to intensify agriculture in a national or global scale is accompanied by increased use of agrochemicals, which would cause unprecedented ecosystem implication, loss of ecosystem services and destruction of natural ecosystems (Tilman, 1999). However, the overall impact of a pesticide depends on its behavior in the environment, eco-toxicity and on the amounts applied. For developing countries like Tanzania, where technology and scientific research advancement is still lagging behind, effective regulatory and policy changes are desired to control environmental impacts of agricultural intensification programs. Modernization and massive agribusiness investments in sensitive ecosystems of Tanzania is a major concern to the scientific community and might require intensive monitoring and management plans in the future.

1.3.6 Pesticide management and status of pesticide use in Tanzania

Tanzania Pesticides Regulations of 1984 (made under section 41 of the Pesticide Act, 1979) seek to safeguard the registration and certification of all pesticides intended to be used in the country, providing licenses to importers or manufacturers, and set criteria for the protection of public health and the environment from pesticide contaminations. According to Tropical Research Institute of Tanzania, (TPRI, 2011) registration and certification of pesticides fall in 4 categories: - (i) Full registration category:-whereby certificate expires within five years. (ii) Provisional registration category: - whereby pesticides are registered for general use in a period of two years. (iii) Restricted registration category: - whereby pesticides are registered for restricted use for two years only and (iv) Experimental registration category: - whereby pesticides are registered for experimental purposes only, although the duration of such certificate is not clearly stated. According to Pesticide Regulations of 1984, restricted certificate is offered when a pesticide is highly toxic, persistent, bioaccumulates or when there is no known or available effective antidote of that pesticide in case poisoning occurs. TPRI mandates all the pesticide importation, certification fees, handling and disposal procedures under the plant protection section of Ministry of Agriculture and Cooperatives. Global industrial and agricultural trade liberalization has accelerated distribution and use of pesticides in areas where food and cash crops are cultivated either in large scale or small scale

(Ecobichon, 2000). In Tanzania, the use of pesticides is emphasized in the recent political declaration, KILIMO KWANZA (a *Swahili* phrase meaning *Agriculture first),* which aims at modernizing agriculture through adoption of green revolution tactics (Kilimo Kwanza, 2009). Remarkably, some of restricted for use or banned pesticides and/or their active ingredients in developed world such as in European Union, Canada and USA are still locally formulated and freely marketed within the developing countries (Ecobichon, 2001; Voss & Schatzle, 1994; Wesseling *et al*., 1997). For instance, pesticides such as alachlor, atrazine, DDT, carbofuran, hexazinone, heptachlor, lindane and many more are still provisionally or fully registered for use in Tanzania (see Appendix 6). However, the choice of a type of pesticide to be used is much influenced by the supplier or pesticide vendors (Ngowi *et al*., 2007), costs and availability of such chemicals in the markets (Ecobichon, 2001) and effectiveness of the pesticide in controlling pests (Viviana Waichman *et al*., 2007). The majority of small scale farmers in Tanzania and in some other developing countries, have insufficient knowledge on issues of chemical safety and management (Ecobichon, 2000), besides being not well informed about the hazards associated with exposure to such chemicals on human health and on environment in general (Ngowi, 2003). Inadequate agricultural extension services, widespread of pesticides in the open markets, lack of basic knowledge on risks of pesticides and negligence by farmers, influence unsafe handling practices, uncontrolled usage, storage and disposal of pesticides in developing countries (Ecobichon, 2001).

In order to tackle challenges of food insecurity and poverty reduction, the Tanzanian government has set a number of initiatives and national developmental strategies to foster a gradual adjustment from small-scale agriculture to large-scale farming. Agricultural expansion in Tanzania is envisioned to be associated with inputs of modern agrochemicals (pesticides, fertilizers) and improved seeds and breeds, mechanization and irrigation schemes (Kilimo Kwanza, 2009). Multiple stressors and cocktail of pesticides and nutrients in the environment following rainfall seasons or flood events in the agrarian systems may result into multiple additive or synergistic effects (Faust *et al*., 2001; Negri *et al*., 2005; Owen *et al*., 2003) which exert much pressure on species sensitivities (Luoma, 1977), structure of the natural communities (Knauer *et al*., 2010) and untargeted organisms (Tadonléké *et al*., 2009; Magnusson *et al*., 2008) thus increasing the risk of ecosystem destruction. Environmental and ecological quality assessment is essential for implementation of monitoring programs that intend to minimize the risk of toxic substances in natural ecosystems. This study focused on the assessment of the potential impact of pesticide contamination in soil-water-sediment matrices of paddy rice and sugarcane agronomic systems in Tanzania. Sample

toxicities were measured using acute and chronic bioassays, such as luminescence inhibition of *V. fischeri*, 72-hour algae growth inhibition test using *P. subcapitata,* inhibition of metabolic activity of yeast, *S. cerevisiae* and bacteria contact assays using *A. globiformis.*

1.3.7 Agronomic systems in Tanzania

Tanzania is rich in land, livestock and other natural resources which are nevertheless insufficiently managed to support agriculture, a backbone of its GDP (World Bank, 2009). Tanzania has warm weather with seasonal variations of temperature according to the geographical location, relief and altitude, rainfall pattern is either monomial or bimodal (URT, 2007). The average duration of the dry season is 5 to 6 months (URT, 2007). The mean annual rainfall varies from 500 millimeters to 2,500 millimeters and above (URT, 2007), depending on topographical locations. In the past one decade, rainfall pattern has become much more unpredictable, with some areas receiving extremely low or high rainfall per year (URT, 2007) due to the impacts of climate change. The coastal regions have adequate rainfall patterns, fertile soils and water resources (MAFC, 2011), with the exception of arid and semi-arid areas (TIC, 2012; World Bank, 2007, 2008). Annual mean temperature ranges from a mean daily temperature of between 24°C and 34°C, but within the plateaus mean daily temperatures range between 21°C and 24°C and in the highland areas temperatures range from 15°C to 20°C (SAGCOT, 2011). Soil´s water holding capacity and different weather pattern form unique agro-ecological zones based on altitude and precipitation pattern, varying from tropical in the coast, to semi-temperate in the mountains and dry season (*Kiangazi*) in the plateau regions (SAGCOT, 2011). Moreover, agricultural production in Tanzania is tremendously dependent on the rain-fed farming and therefore any uncertainty in rainfall patterns contributes to wide variations in crop production. Main food crops and also for trivial economic incomes are maize, sorghum, millet, banana, cassava, rice, beans, sweet potatoes, wheat pulses while main cash crops are coffee, cashew nuts, cotton, sugarcane, tobacco, tea and sisal, as well as spices from Zanzibar (IFAD, 2007). Social economic development and climate change impacts have put multiple pressures on agro-ecosystems, and some studies have highlighted the potential for shifts in agro-ecological zones (Kurukulasuriya & Mendelsohn, 2008). The agricultural sector in Tanzania has not performed according to the government's expectations in terms of contributing towards national economic growth and poverty reduction among smallholder farmers, and has even failed to ensure food self-sufficiency, given the sporadic food shortages in drought years (MAFC- FAO, 2008; TNBC, 2009). Agriculture is almost entirely driven by smallholder farmers who depend on hand hoe, traditional

rain-fed agricultural and animal husbandry practices. It is characterized by limited access to and participation in input and output-markets (FAO, 2011), extension services, knowledge, information and financial services (IFAD, 2007). In sub-Saharan Africa, agriculture is the largest economic activity that sustains the livelihood of millions of people (FAO, 2011), therefore good and reliable agricultural practice is an important feature for food security, employment, poverty reduction and economic development. For any increased population, like in Tanzania, whose economy depends on agriculture (FAO, 2003), the pressure to produce more food per area as well as to reclaim more land for agriculture cannot be avoided. Based on the expected growth of the world population, Hassan *et al*. (2005) have forecasted the increase of demand for food by 50% by year 2030. FAO (2003) has documented that in Sub-Saharan Africa and many developing countries, agriculture and its related industries are essential to economic development and to reducing mass poverty and food insecurity. Agriculture in Tanzania accounts for 45% of GDP and 75% of the population is rural (World Bank, 2009), while the manufacturing sector accounts for about 9.5% (URT, 2009a). The commitment to accelerate economic growth, increase food security and fight poverty has been consistently implemented through a series of long and short-term strategies ranging from sector-specific to multi-sectoral policies. For instance, MKUKUTA (a *Swahili* acronym for the *National Strategy for Growth and Reduction of Poverty)* forms part of Tanzania's efforts to deliver on its national Vision 2025. Renewed commitment to agriculture under MKUKUTA II is thus intended to achieve the twin objectives of increased agricultural production and reduction in hunger/food poverty (URT, 2010; NSGRP, 2010). Consequently, the agricultural sector emphasizes the necessity to expand crop production to all fertile lands in Tanzania, including flood plains and wetlands where farming can continue during drought seasons, but environmental conservation is seldom integrated into such plans. The current agricultural expansion and large-scale investment initiatives in Tanzania have not entailed sufficient efforts to foster agriculture under sustainable watershed management. It is very probable that the current agricultural strategies of the Tanzanian government to increase food security and to combat poverty will put much pressure on river basins and wetlands ecosystems. There is a need to address political, socio-ecological and environmental challenges of such investments, because environmental and ecological safeguarding is rarely integrated into the economic development plans.

1.3.8 Potential of wetlands resources for livelihood

Wetlands provide various ecosystem services for the wellbeing of the surrounding communities, such as improved water quality, groundwater recharge, shoreline anchoring, flood control and support a diverse variety of fish, wildlife and plants (MEA, 2005). The nature of dependence and demands on wetlands resources are site specific and vary from socio-economic status of people within the same community (Rebelo *et al*., 2010; Schuyt, 2005). In the developed world, wetlands are maintained for recreational and or education purposes (Bergstrom *et al*., 1990; Boyer & Polasky, 2004), or are reclaimed for developmental programs (Wolff, 1992) or for agricultural activities and other land uses (Baldock, 1984; Kracauer Hartig *et al*., 1997). In poor or developing countries, wetlands are ordinarily used as a source of water, food, building materials or for tourism activities for revenue incomes and sometimes are reclaimed for public projects. However, the extent of utilization of and dependence on wetlands ecosystem services for livelihood in developing countries depend on the level of education or socio-economic situation of the community (Nabahungu & Visser, 2011), resource availability and land ownership (Adger & Luttrell, 2000), natural setting of the wetland i.e. location or accessibility of the wetlands (Mwakaje, 2009), and socio-cultural, historical, national and political perspectives (Silvius *et al*., 2000). A number of studies have indicated that a significant proportion of people in developing countries depend upon the use of wetland resources in one way or another for their livelihoods. For instance, studies in southern and eastern Africa have revealed that wetlands and their surrounding catchments sustain rural livelihood through provision of a large range of natural resources such as food, fresh water, plant-herbs, construction materials and wildlife (Jogo & Hassan, 2010; McCartney & Van Koppen, 2004b; Turpie, 2000). Despite the value and importance of these services for many people in developing world, wetlands ecosystems are threatened by unsustainable agricultural practices and insufficient political, social and ecological management plans (Falkenmark *et al*., 2007; Finlayson, 2007). Long term integrity of wetlands ecosystem is stated in the wise use concept of Ramsar Convention, which defines sustainable use of wetlands as “human use of a wetland so that it may yield the greatest continuous benefit to present generations while maintaining its potential to meet the needs and aspirations of future generations” (Ramsar Convention Bureau, 2000). In Sub-Saharan Africa, wetland agriculture is a major pursuit among rural communities; since it provides suitable cultivation conditions for perennial food crops throughout the year (Adams, 1993), thus making wetlands a significant factor in food security and income generation (Wood, 1996). A number of studies have shown that more than 60% of household income of communities

surrounding rural wetlands is generated from wetland cultivation in Africa (Adekola *et al*., 2008; de Groot *et al*., 2002; Nabahungu & Visser, 2011; Rebelo *et al*., 2010).

1.3.9 Wetland management in Tanzania

The Wildlife Division of the Ministry of Natural Resources and Tourism manages all wetlands in Tanzania (MNRT, 2004a). The whole management approach from community participation to the national level is molded by multi-sectoral organs which have diverse objectives, goals, administration and specialized legislation (Mombo *et al*., 2011). Therefore, there is no one specific legal and policy framework for wetlands (MNRT, 2004b). Rather, wetland management issues are regulated by contradicting policies such as those covering agriculture, forests, water, mining, wildlife and environment, which do not precisely and directly address wetlands protection in their provisions. For example, while the agricultural policy promotes intensive and large-scale mechanized farming systems to increase food and cash crop production, the fishery policy encourages improved commercial fishing with little concern for the environmental consequences, and the investment policy emphasizes further investment in various sectors to promote economic growth without taking into account environmental measures such as environmental impact assessments (EIA). The whole issue of wetlands management in Tanzania is therefore regulated by fragmented policies and management strategies whose implementation depends on political, sectoral, regional or district interests. According to Turner and Jones (2013) when inter-related sectors are governed by different specific policies and/or are overseen by different government departments, complex policy overlaps and inconsistencies are bound to arise, a situation which is affecting wetland management in Tanzania today. A brief of wetland management policy fragmentation and overlaps in Tanzania is discussed below:

Wildlife sector: The wildlife sub-sector addresses the sustainable conservation of wildlife and wetlands resources in Tanzania. Although the Wildlife Policy of 2007 and the Wildlife Conservation Act of 2009 have adopted the definition of wetlands from the Ramsar Convention (URT, 2009c), only wetland areas reserved for wildlife located within a national parks or game reserves are protected under this jurisdiction. The Act, outlaws crop cultivation or agricultural usage in any wetland reserve areas s. 18(2), 8(4) and 20(1(c).

Agriculture sector: Agriculture and Livestock Policy of 1997 permits the exploitation of water resources such as big and small rivers, lakes and groundwater for the development of irrigation

systems necessary for food production, s.5 (E(1). Pesticides Regulations of 1984 (made under section 41 of the Pesticide Act, 1979) seek to safeguard the registration of all pesticides intended to be used in the country, providing licenses to importers or manufacturers, and set criteria for the protection of public health and the environment from pesticide contaminations. However, neither the Agriculture and Livestock Policy nor the Pesticide Regulations sets criteria to restrict or plan for monitoring pesticide use in sensitive ecosystems; neither do they set limits of agrochemicals discharge from runoffs to minimize its impact on wetlands and downstream river basins.

Land sector: The Tanzanian Land Policy of 1999 categorizes a `wetland´ as unproductive and hazardous land, useless for economic development and is by law not allowed to be allocated to any individual or group of individuals for whatever use. The policy promotes land tenure systems that facilitate social and economic development without disturbing the environment's ecological balance. Additionally, the Land Act of 1999 (as amended in 2004) as well as the Village Land Act of 1999 also categorize wetlands as `hazardous land´ where development is likely to pose a danger to human life or lead to environmental degradation. In general, under Tanzanian land policy and legislation, wetlands are considered to be of no economic value. Consequently, no clear wetland management plans are mentioned.

Forestry sector: The National Forest Policy of 1998 promotes protection of mangroves and swamp forests, which have important functions in water catchment areas. It sets out general guidelines for sustainable supply of forest products and services, and acknowledges the necessity for cross-sectoral coordination for forest conservation. It also identifies the need for reforms to reflect its objectives and strategies to take into consideration the protection of water sources such as rivers, streams, wells and wetlands (s. 4(3(2)) and to involve local communities and other stakeholders in encouraging watershed management through research and information dissemination. In line with this policy, one of the objectives of the Tanzania Forest Act of 2002 is to ensure ecosystem stability through conservation of forest biodiversity, water catchments and soil fertility. In this Act, "water catchment" is referred to as one of the sensitive areas to be managed. Consequently, s. 18(1) of the Act requires that Environmental Impact Assessments must be conducted in respect of all developmental projects to be implemented in sensitive forest areas such as watersheds. The protection of watersheds or wild plants is declared to be protected as a local authority forest reserve only if authorized by the Minister (s. 22(4(b)). However, there is no further regulation on

the protection of wetlands and other river catchment areas, which do not fall within the areas of forest reserve.

Mining sector: The Mineral Policy of 1997 specifically mentions the risk of the environmental degradation and recognizes the need to integrate environmental and social concerns into mineral exploitation development programs (s. 3(3(12(1)). Through this policy, the role of the government is to set appropriate guideline for mining in the restricted areas such as forests, national parks, water sources and other designated areas like wetlands. The Mining Act (1998) under s. 100 (2) (d) and (j) requires mining license holders to take all appropriate measures for the protection of the environment and water, including wetlands by eliminating all adverse effects from mining operations. No further regulation or specific guidelines are provided by this Act as to how wetlands and river basins should be protected.

Fishery sector: The National Fisheries Policy and Strategy Statement of 1997 promote protection of biological diversity of fragile coastal and aquatic ecosystems by preventing pollution and habitat destruction (s. 3(3(6)). The policy statement promotes the wise use principle by acknowledging the economic significance of wetlands. However, it is more concerned with the management of coastal than with inland wetlands. Pursuant to section 44 of the Fisheries Act No. 22 of 2003 and section 40 of the Regulations issued under s. 57(2(k) of the Act, it is an offence to possess poison within the vicinity of any water body containing fish, or to use any poisonous chemical or toxic substance for purposes of fishing. In this Act, ''poison'' means any natural or synthetic chemical used to kill fish. The fishery Regulations also prohibit any person to cause water pollution in any lake, river, dam, estuary or seawater by releasing into water any solid, liquid or gaseous poisonous material(s. 41). Although the Act and its Regulations restrict the use of poison near water bodies that contain fish, they show no concern about the impact of pesticide agricultural runoff to the water bodies and wetlands as breeding sites for fish.

Water sector: The National Water Policy of 2007 recognizes wetlands as one of the water resources, and it seeks, among other objectives, to improve the management system and conservation of ecosystems and wetlands. The policy emphasizes the importance of wetlands in maintaining hydrological cycle, flood control, sediment retention and nutrient recycling, and microclimate stabilization. It also highlights how agricultural activities contribute to agrochemicals pollution to water bodies especially during runoff. However, with regard to economic planning of

water uses, this policy assigns the sustainable environmental and ecosystem protection as second priority, while human consumption as a basic need is of highest priority. For sustainable water and conservation, the policy aims at having in place appropriate principles and procedures for managing water quality, conservation of water resources and protection of ecological systems such as wetlands, floodplains, estuaries and coastal zones. It, however, does not state such principles or procedures. In s. 4(9), the policy mentions wetlands and catchment degradation as one of the challenges, which require specific strategies and actions during development, and management of water resources.

The Water Resource Management Act No. 11 of 2009 was enacted subsequent to the water policy. Among other objectives, the Act seeks to ensure protection and conservation of nation´s water resource, protecting biological diversity especially the aquatic ecosystems. Pursuant to s. 37(1) of the Act, the Minister responsible for water has the mandate to establish protected zones of any catchment, swamp, reservoir, wetlands, springs or any other sources of water. However, neither the policy nor the Act clearly addresses penalties or sanctions associated with wetland destruction.

In Environmental sector: The National Environment Policy of 1997 aims at improving environmental management and conservation of biodiversity and wetlands. It elaborates clearly how improving food security and eradication of rural poverty through agriculture can lead to the encroachment in public lands including wetlands and woodlands. The policy objective regarding agriculture, (s. 46) is to control run-off of agrochemicals to minimize pollution of both surface and ground water. Under s. 16, the policy recognizes that any expansion in agriculture implies bringing more land into production from existing forests and woodlands, wildlife areas; draining wetlands; expanding irrigated agriculture, and / or increasing the use of agrochemicals and threatening the quality of surface and groundwater. It also elaborates the consequence of poverty on environmental degradation (s. 21), and emphasizes the necessity for sectoral policies to address poverty issues in relation to sustainable resource utilization. This policy was formulated apparently to unify natural resources policy to engage the contradictions, minimize sectoral conflicts and an overlap of activities in respect of natural resources management. The policy in s. 48 addresses, among other issues, management of wetlands under water and sanitation section. The revised Environment Management Act No. 20 of 2004 provides legal and institutional framework for sustainable management of the environment. It also recognizes wetlands as fragile ecosystems, which play an

important role in water systems. The Act mandates the National Environmental Management Council (NEMC), which was established in 1983, to oversee the management of all natural resources. NEMC, which is under the Vice President's Office, is tasked with the role to coordinate, raise awareness and provide advice on all matters pertaining law enforcement on environmental conservation, management and sustainable use of the natural resources in Tanzania.

General observations

The conversion of sectoral policies such as those identified above into legislation sometimes takes extended time, especially if the envisioned legislation does not mirror political interests of some decision makers. As local policies are not laws, they are not enforceable in courts, nevertheless Tanzania is obliged to adhere to the guidelines of Ramsar Convention to which is a signatory. Moreover, the effectiveness of the above legislation in protecting wetland ecosystems is weak because of poor or non-enforcement, low or lack of sanctions, which respond adequately to the actual threat. Lack of designated institutions to handle wetland issues, limited staffing, inadequate government support, fragmented policies and inadequate laboratories for scientific analysis are some of the major challenges for sustainable wetland management in Tanzania.

1.3.10 Tanzania and the Ramsar Convention: Integrated land use

The Ramsar Convention recognizes the right of the contracting parties to establish their national wetland policies in a way that is appropriate to their respective national situations (MNRT, 2004a). According to Davis (1993), the mission of the Convention is "the conservation and wise use of all wetlands through local, regional and national actions and international cooperation as a contribution towards achieving sustainable development through the world". The wise use of wetlands refers to how the sustainable utilization for the benefit of humankind coincides with the maintenance of the natural properties of the ecosystem. The Convention underlines the need to promote wise use of all wetlands through local communities and participation in management issues. Since its ratification in year 2000, four Ramsar sites have already been designated under this Convention, namely, the Malagarasi-Muyovozi Ramsar Site in 2004; the Kilombero Valley Flood Plain Ramsar Site in 2002; the Lake Natron Basin Ramsar Site in 2001; and the Rufiji-Mafia-Kilwa Marine Ramsar Site in 2004. These Ramsar sites are facing different threats depending on location, natural resource base and attraction for an economic investment, which attentively seem to support the government to fulfill its Vision 2015. The major threats associated with these four

Ramsar wetlands are briefly elaborated in order to provide an overview of forecasted degradation and unsustainable use of wetlands in Tanzania.

Kilombero Valley Ramsar Site: This is the largest seasonal freshwater lowland floodplain in East Africa located between Kilombero and Ulanga districts of Tanzania (Ramsar, 2012). Destruction of biodiversity hotspot and habitat for endemic species of flora and fauna such as birds, fish and large mammals is a major threat to Kilombero Valley (MNRT, 2004a). As indicated above, the Tanzanian government has earmarked the country´s southern highlands, where this Ramsar site is located under SAGCOT project. This is a project, which entails large-scale agricultural investments and long-term land lease to private investors for purposes of mechanized agriculture. This move in turn means a long-term projection threat to sustainability of this Ramsar wetland. Currently more than 50% of the flood plain has already been converted into agricultural land (TAWIRI, 2008; 2009) by both large and small-scale farming, a condition that contradicts the `wise use´ concept of the Ramsar Convention. The increased agro-pastoral activities and mechanized agriculture that involve intensive use of pesticides causes downstream river basin contamination and destruct fish spawning grounds. Thus, large-scale agriculture will always continue to threaten the integrity of this sensitive and diverse ecosystem. The current trend of unrestricted pesticide use, lack of proper monitoring of the pesticide utilization and insufficient knowledge to local people on the impact of agrochemicals to human health and environment, are evidence of the severe destruction and unsustainable use of this sensitive ecosystem. Therefore, there is a necessity to conduct intensive study on the risk assessment of the impacts of such agrochemicals on the wetland ecosystem and to human health in general in order to recommend formulation of policy and legal frameworks for sustainable wetlands management in Tanzania.

Lake Natron Basin Ramsar Site: It is a rift valley soda lake of East Africa, which covers an area of 224,781 ha basin in northern Tanzania (Ramsar, 2012). The site is known to be the only regular breeding area for the 2 to 4 million Lesser flamingo (Ramsar, 2012). This Ramsar site has unfertile soils and inadequate rainfall to support agricultural activities (URT, 2009b), thereby making food insecurity major socio-economic challenge in the area (NORCONSULT, 2007). Nevertheless, the major threat on Lake Natron Ramsar site is the lately politically approved soda ash facility, which is estimated to mine about 1 million tons of soda ash per year in a lease of 50 years (Bird Life International, 2013). According to Ramsar Advisory Mission (RAM) Report of 2008, Lake Natron in the rift valley zone is estimated to have reserves of at least 4.7 billion cubic liters of soda ash

(sodium carbonate) which is a key raw material for glass, chemicals, soap and detergents. The motivation for the project implementation downplays the fears of lesser flamingo population habitat destruction, and fragmentation.

Rufiji-Mafia-Kilwa Ramsar Site: The Rufiji Basin covers nearly one fifth of the country, and its river tributaries have their origin in the southern highlands, the highest and wettest parts of Tanzania (URT, 2011) where the SAGGOT project is going to be implemented. This is the only coastal marine Ramsar site in Tanzania which has the greatest concentrations of well-developed coral reefs (Ramsar, 2008) and mangrove forests (Matiza & Chabwela, 1992; URT, 2009b). Poverty is rampant (Hogan *et al*., 2000), with about 150,000 people depending on unsustainable agricultural and fishing practices for their living (UNDP, 2012; WWF, 2009). The major threat to this freshwater and marine Ramsar site is coral mining (Darwall *et al*., 2000), coastal erosion, and degradation of marine habitats and fisheries production (WWF, 2009, 2010). The sustainability of this diverse coastal-marine Ramsar ecosystem is also threatened by current oil and gas exploration activities following recent discovery of gas and oil in Tanzania's coastal areas (IRG, 2008).

Malagarasi-Muyovozi Ramsar Site: This Ramsar site covers vast area (Ramsar, 2000) and its sensitive areas are very remote with poor accessibility (DANIDA, 1999) where management, conservation and developmental programs are a challenge. The major threat to this site is the conversion of wetland areas into agricultural land and accompanying land degradation related to the cultivation of tobacco by the surrounding communities (DANIDA, 1999).

1.4 Objectives of the study

Eco-toxicological studies, which range from biochemical mechanisms of toxicity of individual toxicants to combined effects on populations and communities caused by toxic chemicals, are very limited in Tanzania. Chronic or acute toxicity tests are typically conducted in pharmacology and veterinary or human medicine research fields to establish potential risk of the certain observed consequence. Environmental activists and conservationists in Tanzania rely much on the observed degradation trends or observed physical deformation or mortality due to toxicants released into the environment. Sometimes, unobserved impacts are overlooked, thus, require a proof of scientific research to convince policy makers to initiate management or protection plans. Wetland ecosystems and other biodiversity hotspots in Tanzania are under increasing threat of habitat degradation from population growth, agricultural expansion and its associated agribusiness

investments, mining activities and other developmental projects that release or might lead to the release of toxic or non-toxic substances into the environment. There are no documented researches in eco-toxicology using bioassays for Tanzanian environments. Large-scale agricultural activities require immediate research to address the field of eco-toxicology in order to provide an insight of ecological risk assessment to the policy makers for monitoring programs and environmental regulations issues. This study focused on assessing the suitability of using a battery of biotests developed in the temperate countries for assessing pesticide contamination in soil-water-sediment in Kilombero Ramsar wetland in Tanzania.

The following specific objectives are addressed in this study:

1. Application of three standardized bioassays for temperate regions to determine eco-toxicity of water, soil and sediment samples of freshwater tropical wetlands, Kilombero valley Ramsar site
2. Critical discussion on suitability of temperate biotest batteries for assessing pesticide contamination in tropical agronomic systems
3. Estimation of potential risks to humans and aquatic life based on chemical analysis data of soil-sediment-water samples from Kilombero valley Ramsar Site agricultural fields.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Chapter introduction

This chapter details the various procedures and methods used to address the objectives of this study. Three standardized bioassays were selected. (i) Algae Growth Inhibition Test (AGI) using *Pseudokirchneriella subcapitata,* a green algae which is sensitive to environmental contaminants such as herbicides and used as a representative species of primary producers in the plankton community, thus a good indicator for aquatic environment risk assessment studies. (ii) Bioluminescence Inhibition Test (LBT) using *Vibrio fischeri,* a marine bacterium which emits luminescence during metabolic activity. This bacterium represents the group of destruents or decomposers. The metabolic activity of *V. fischeri* can be inhibited by various toxicants thus a good indicator for risk assessment studies especially in screening of toxicities of contaminated samples. (iii) Bacteria Contact Assay (BCA), using *Arthrobacter globiformis,* a soil bacterium with an affinity to surfaces. This bacterium is a good assessment tool and indicator for contaminated solids. This test was preferred in order to assess direct exposure of organisms to contaminated sediments or to particle bound contaminants. Moreover, other two non-standardized biotest were optimized and used in this study. (i) Acute Algae Test (AAT) using *Pseudokirchneriella subcapitata.* This test evaluates the responses observed in the AGI after exposure to environmental samples. Since the conventional 72-hr AGI test involves the impact on multiple cell generations then the short-term test was developed to determine effects of tested chemicals or samples on one cell generation and the state of electron transfer within PS II. (ii) Yeast Test (YT) using baker's yeast, *Saccharomyces cerevisiae,* widely distributed fungi, which grow under varying ecological conditions and it displays most of the features of higher eukaryotes. This test was conducted in order to examine environmental impact of fungicides.

The aforementioned arrays of biotests were applied on Tanzanian sediment, soils and water samples to investigate their applicability in the tropical agronomic systems. Qualitative questionnaire surveys and face-to-face interviews were conducted in the Kilombero valley plantations in order to get an overview of the status of agrochemicals used in the study area and other relevant information. Subsequently sediment, soils and sediment samples were collected during dry and rainy seasons. Meteorological data on temperature, rainfall pattern, solar radiation and intensity, soil temperature, relative humidity and wind speed were also collected during

sampling seasons. In order to integrate the toxicity responses of the bioassay results in a comprehensive risk assessment, analytical parameters such as sample C/N ratios, grain size, trace and heavy metals screening, pesticide screening and other geo-physical parameters were carried out. All bioassay laboratory procedures were conducted at Applied Aquatic Toxicology laboratory of Hamburg University of Applied Sciences.

2.2 Preliminary field surveys

Research permit and introductory letter was pursued from Sokoine University of Agriculture-Office of Vice Chancellor. A preliminary field survey /observations were conducted in January 2012. The aim of the research was familiarized to the management of the study companies. A qualitative questionnaire on status of agrochemicals usage in the plantations was administered to agricultural officers or company agronomists of 8 companies or plantations that practice large-scale crop plantation in the Kilombero Valley Ramsar site (KVRS). This questionnaire was made available to the plantation one week in advance, so that all detail information could be provided. Appendix 1 provides details of information gathered by the questionnaire. Furthermore, a face-to-face interview was conducted to plantation managers or a company´s spokesperson, in order to reveal any other critical information, which could not be provided freely in the questionnaire. Any precarious information during the survey was treated anonymously. Preliminary visits were done in 8 plantations/farms (Fig. 2.1), which practice large-scale farming of rice and sugarcane as described below.

1. Illovo-Kilombero Sugarcane Plantation Company and its sugarcane fields which is located at the very north of the Ramsar site in Kidatu. The company owns about 6,000 ha of land in the Msolwa River valley sub catchment, which adjoins main Ruaha River downstream. The company also receives sugarcane from the out-grower schemes, about 7,000 ha that are located in the Ramsar site.
2. Kilombero Rice Plantations Ltd in Mngeta farms which owns about 10,000 ha of paddy and irrigated rice.
3. Idete Agricultural Prison in Idete, which grows rice using prisoners as laborers.
4. Mbingu Sisters farms in Mbingu, which owns about 3,000 acres of total land and produce paddy rice, maize, bananas and sunflower from the area of 400 acres.
5. Kiberege Agricultural prison farms in Kiberege, which grows both rice and sugarcane using prisoners as laborers.

6. Tanganyika Agricultural Centre-Katrin farms which owns about 200 acres for experimental and research paddy rice production.
7. Chita-National services and agricultural farms for paddy and irrigated rice.
8. Kilombero Valley Teak plantations Company that owns about 28,159 ha of land on a 99-year lease. Some part of plantation is within the Ramsar Site, although the major part of timber farms are located in Mavimba village, Ulanga districts in the Mahenge highlands outside the Ramsar site.

The initial information gathered were type of crop and size of the land occupied for agriculture, duration of agricultural practices, type, method of application and estimated quantities of agrochemicals used, general knowledge on the impacts of agrochemicals in aquatic ecosystem. Besides, in order to associate the information of pesticide use, dissipation and environmental factors, available environmental or weather data records collected by companies, such as daily precipitation, soil moisture, wind and solar radiation were also incurred.

2.3 Description of study area and sampling designs

2.3.1 Geographical location and geo-physical features

This study was conducted in the largest low-altitude freshwater wetland in East Africa (MNRT, 2004a). The Kilombero Valley flood plain Ramsar site is located in the conserved eastern Arc mountains of Tanzania, between the Udzungwa catchment mountains reserve in the north-west (7°47'S, 36°36'E) and the Mahenge escarpment in the southern east (8°45'S, 36°39'E) (Ramsar, 2002). Kilombero Valley was designated a Ramsar site in 2002, two years after Tanzania had ratified the Ramsar Convention. The Ramsar site central coordinates points are 8°40'S and 36°10'E which covers an area of 796,735 ha with a wetland catchment area of 40,000 km^2 (Ramsar, 2002). The area has numerous temporal and permanent rivers, which serve as a source of freshwater and livelihood activities for surrounding communities. All the complex streams of river channels (Fig. 2.1) drains into the permanently flowing Kilombero river, which flows over the Ramsar site for 65 km downstream to confluence with the Luwegu river in the Rufiji river delta, that finally drains to the Indian Ocean. The valley is sub-humid with a mean annual temperature of 26°C and a mean annual total rainfall of 1,600 mm (Bakengesa *et al*., 2011). It has a bimodal rainfall pattern with short rains (light and sporadic) from December to February and long rains (heavy and regular downpour) from March to May or June (Bakengesa *et al*., 2011). Flooding peaks between March

and April convey important nutrients to the valley leading to highly inundated fertile and productive soils (SAGCOT, 2011).

2.3.2 Samples collection, preservation, handling and storage

Surface sediment, soil and water samples were collected during rainy and dry seasons in 8 sugarcane rice and teak plantations in the KVRS (Fig.2.1). The rainy season comprised of April 2012, while the dry season included January 2013. A total number of 143 samples (58 water, 68 sediments and 17 soils) were collected in all sampling stations for the two seasons. Fine sediment fractions were collected either directly from the surface banks or beneath a shallow sluggish aqueous layer using a stainless steel spoon. Water samples were collected by using previously acid washed plastic bottles either adjacent or at the same sites. During dry season, representative soil samples were randomly collected from newly ploughed or planted rice or sugarcane agricultural fields with areas of variability within the field avoided for the purpose of the study objectives. Following collection, sediments and soil samples were transferred to the previously acid washed dry polythene plastic bottles. Samples were preserved in the ice box during the whole period of sampling, and later transported to the laboratory of Applied Aquatic Toxicology, Hamburg University of Applied Sciences, Germany. On site temperatures and pH values of sediment and water samples were recorded, while salinity, redox potential, dry weight and organic content were measured in the laboratory before further handling.

Ammonium, nitrite, nitrate and phosphate ions in the water samples were measured on site by using reflectometry (RQflex®10) at different detection limits of test strips.

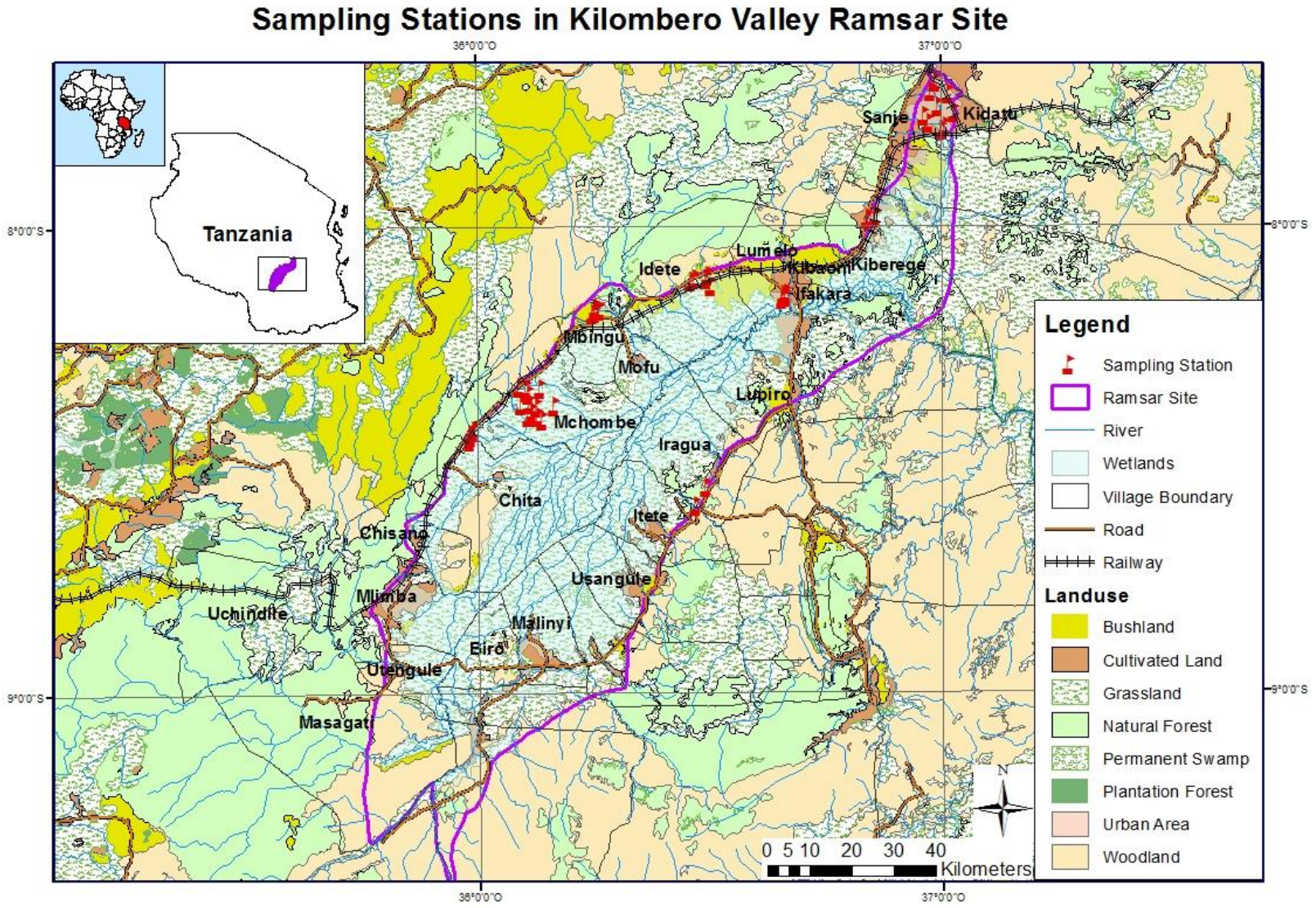


Figure 2.1: A map of Tanzania showing location of Kilombero Ramsar Site and sampling stations.

2.3.3 Quality assurance

All sampling bottles were pre-cleaned with tap water and laboratory detergent and soaked overnight in an acid bath (3M HCl), rinsed thoroughly 3 times with deionized water, air dried and packed in a big black polyethylene plastic to avoid contamination. At the time of collection, each sealed sample bottle had labels affixed to it which portrayed-sample station, sort code, date of sampling and initials of sample matrix. pH and temperature of sediment and water samples were directly measured at the time of collection using calibrated pH meter with temperature probe. Samples were preserved at the sampling site in an ice chest containing several cold packs before refrigerated at 4°C on the last day of sampling followed by one day holding time before transported to Germany by using ice chest box. Samples were stored at 4°C to minimize microbial activities, supplemented by 2 to 3 weeks holding time for bioassays analyses, and afterwards deep frozen until analytic assessment were conducted. At the time of collection, GPS coordinates of each sample and all relevant sample information were entered into a field log book. All biological data generated from biological measurements were validated based on specific test acceptability criteria, precision of measured duplicates, representativeness, comparability, and completeness. The data were evaluated against reference values for positive and negative controls and lastly uploaded in the central database.

2.4 Analytical assessments

2.4.1 C/N nutrients analysis

Sample preparation

Soil and sediment samples were dried at 105°C for 24 hours. In order to facilitate mutual compaction of different digestion procedures, the samples were homogenized in a ball-mill into a fine powder (<100 µm). All powdered samples were stored in small air-tight plastic containers.

Total C and N in samples were determined using a CHN Perkin Elmer 2400 Elemental Analyzer. Using Tin capsules, about 20 mg range of dry-powdered standards, soil and sediment samples were weighed, compactly wrapped and placed in a clean 96 well storage tray until further measurements were performed. All the weighing utensils and glass plates were cleaned with water and acetone. Two replicates of each sample and the standards were wrapped in a tin-membrane paper, then digested in a C/N analyzer. All tools were cleaned after replica of each sample or blank or standard to avoid cross contamination. The standards were kept in airtight, dark bottles to minimize exposure to light and air. The analyzer instrument was set to equilibrate for about 2.5 hours until

the combustion and reduction temperature of 980°C and 640°C respectively were reached. Before sample measurement, the instrument was calibrated with blanks until the preferred precision was achieved.

2.4.2 Determination of sample potential acidity (pH)

About 20 g of wet sediment and soil samples were left open in clean petri-dishes to dry. Samples were sieved <2mm to remove stones and plant debris, and mixed thoroughly to obtain a representative sample. pH values of soil and sediments were determined by mixing 10 g of air dried samples with 25 mL of freshly prepared 0.01 M calcium chloride. Essentially, Ca^{2+} ions replace some of the H^{+} ions from the soil particles, coercing the hydrogen ions into the solution and making their concentration in the bulk solution higher to those found in the field. The mixture of samples and calcium chloride was kept standing for one hour while stirring gently with glass rod in every 10 minutes before measuring of pH values using a digital pH meter with a glass electrode. Each suspension was stirred vigorously using a glass rod immediately prior any measurement and three pH replicate values were recorded.

2.4.3 Determination of soil electrical conductivity (EC)

In this study, the determination of sample salinity was considered of great importance, because natural soils contain different amounts of dissolved ions such as Na^{+}, Mg^{2+}, Ca^{2+}, K^{+}, Cl^{-}, SO_4^{2-}, HCO_3^{-} or CO_3^{2-} which can be altered by agricultural practices and thus influence soil salinity. In order to reduce the extent of altering physical properties of samples, air drying was favored as opposed to oven drying at higher temperatures. Cohesive soils were crushed by pestle before sieved to <2 mm size. A mixture of 5 g of air-dried, soil/sediment samples with 50 mL of de-ionized water was horizontally shaken by an electrical shaker in an Erlenmeyer flask for 1 hour. Electrical conductivity of unfiltered supernatant was measured using potassium chloride (KCL)- calibrated EC-meter into mill-Siemens per centimeter (mS/cm). The % salinity was calculated in reference to the EC of the KCL-calibration solution (0.528 mS/cm at 25°C), i.e. Salinity in % (calculated as KCL) = measured electrical conductivity in mS/cm multiplied by 0.528 factor.

2.4.4 Dry weight and grain size analysis

Known weights of each wet sample in an aluminum weighing tin were oven-dried to constant weight at 105°C for 24 hours. The samples were cooled in a desiccator, reweighed and percentage dry weights were calculated using the following formula:

$$\%\ Dry\ weight = \frac{W2 - W1}{W3 - W1} \times 100$$

Where: $W1 = Weight\ of\ tin\ (g)$

$$W2 = Weight\ of\ dried\ soil + tin(g)$$

$$W3 = Weight\ of\ moist\ soil + tin\ (g)$$

Grain size analysis was performed by GBA *(Gesellschaft für Bioanalytik mbH)* which employ the procedural and methods described by *Deutsches Institut für Normung e.V* (German National Standard) DIN 18123 (2011).

2.4.5 Pesticide screening

Screening of pesticides from sediment, soil and water samples was conducted by GBA (*Gesellschaft für Bioanalytic mbH)*-Hamburg using DIN-ISO standard. For glyphosate and its major metabolite, amino-methyl-phosphonic acid (AMPA), ISO 21458 (2008)-(Liquid-extraction, LC-MS/MS) was employed with lowest detection limit of 0.01 µg/L. All other pesticides results from this study were determined according to DIN 38407-35 (2010)-German standard methods for the examination of water, waste water and sludge-(jointly determinable substances group, F) using high performance liquid chromatography and mass spectrometric (HPLC_MS/MS), under different detection limit according to type of matrix and type of pesticide intended for detection.

2.4.6 Heavy metal and trace elements screening

2.4.6.1 X-Ray Fluorescence Analysis (XRFA)

Fine powdered sediment and soil samples (<100 µm) were prepared according to Section 2.4.1. Major and trace metals were screened in the powdered and homogenized samples by using Handheld XRF Analyzer, Delta Premium portable XRF (Olympus Innov-X, Waltham, MA) from Olympus GmbH.

Principally, an XRF analyzer consists of three major components: (i) a radiation source that generates X-rays (a radioisotope or x-ray tube) which was Ta-AU X-ray tube at 10 - 40 kV (ii) a detector that converts X-rays emitted from the sample into measurable electronic signals (iii) a data processing unit that records the emission or fluorescence energy signals and calculates the elemental concentrations in the sample. Elemental quantification was made via integrated ultra-high resolution silicon drift detector (SDD). Scanning occurred as a set of 2 beams each set to scan for 30 seconds. Standard reference material, a standard containing certified amounts of metals in soils, rock or sediments was used to associate the accuracy of the method and the obtained results.

Before starting measurement, the instrument was calibrated by using Stainless Steel Calibration Check Reference provided with the instrument. Three measurements of each homogenized sample were taken. Standardized Reference Material (SRM)®2711a, certified by USEPA-National Institute of Standards and Technology for 25 elements was used to ascertain the results obtained by portable XRFA. The SRM was intended for soils, sediments or other materials in the dried, powdered matrix. A portion of dry homogenized fine powdered SRM was measured directly using XRFA. The concentrations of the detected metals in mg/kg were used to calculate percentage recovery by comparing with the SRM certified values.

2.4.6.2 Inductively coupled plasma optical emission spectrometry (ICP-OES)

Aqua regia extraction as described by the German standard DIN 38414-7 (1983) using HNO_3, HCL and H_2O_2 was performed for multi-elemental determination in the soil and sediment samples. Preliminarily, all glassware and flasks used in the experiment were pre-cleaned with detergent, thoroughly rinsed with distilled water and immersed in HNO_3 (10% v/v) overnight. Afterwards, all materials were carefully rinsed with deionized water and left to dry in a laminar fume hood to avoid dust accumulation. An analytical linear calibration curve for multi-elemental standard was prepared after successive dilutions of the standard stock solution (1000 mg L^{-1}). Dried soil samples (200 - 300 mg in triplicate) were weighed and transferred to digester tubes. In each tube mineral acids (3 mL of 65% HNO_3, 3 mL of 37% HCl and 500 µL of 30% H_2O_2 were added. A pre-digestion step was conducted leaving the mixture in a fume hood at room temperature overnight. The microwave digestion at 120°C was performed for 45 minutes and the digester tubes were kept in a fume hood to cool at room temperature. 500 µL of H_20_2 were further admitted to each digester tube before the extracts were filtered into decontaminated perfluoralkoxy (PFA) plastic bottles. The clear solutions were made up to 50 mL volume mark of plastic measuring cylinders using deionized water. Aqueous solution was screened for metals by using ICP-OES (Perkin Elmer Optima 3300 DV ICP-OES 60-Plasma spectrometer) with reference to multi-elemental analytical standard solution, which was used to calibrate the instrument at different concentration ranges and wavelengths of each analyte. Samples were diluted 1:10 and 1:100 in order to be analyzed within the working range of the method. Three blanks and two certified reference materials (CRM) were used to establish the detection limit of the analytes. The details of the CRMs were as follows:

- Reference Material 1 (CRM-1)-Flood sediment material-certified for Fe, Mn, Cu, Cd, Pb and As, by Friedrich-Schiller Universität Jena, Institute of Inorganic and Analytical Chemistry.

- Standard Reference Material 2 (CRM-2)-Loam soils- certified for Mn, Cu, Pb, Cd and As, by Analytika Co.Ltd, Czech Republic.

The correctness of the calibration was verified regularly after every 15 samples, by a digested certified reference material. Blank solutions were also continued to be analyzed at regular intervals in order to avoid potential carry-over. The XRFA results of selected heavy metals (homogeneous fine dust samples) were compared with ICP-OES results, which were obtained from aqua-regia, digested samples.

2.5 Eco-toxicological assessments

2.5.1. Elutriate preparation

Elutriates were prepared in a sediment-to-water ratio of 1:4 on a volume basis, at room temperature and shaken for 24 hours at 70 rpm according to USEPA (1991). The slurries were then centrifuged at 10,000 g for 20 minutes to obtain a clear supernatant. pH values of supernatants or elutriates were recorded before used in the test.

For Algae growth inhibition test, either the original water samples or elutriates were diluted by using bi-distilled water into four serially diluted concentrations of 1:1, 1:2, 1:4, 1:8, 1:16 whereby the percentage of sample concentration in the test medium were: 80, 50, 25, 12.5, 6.25 respectively.

For luminescent bacteria test: After centrifugation, sediment or soil elutriates and water samples were first adjusted to pH of 7.0 ± 0.2 (using NaOH or HCl) and salinity of 2.0 to 2.2% (using NaCl) for marine bacterium. Then elutriates were serially diluted with artificial seawater-ASW (see Appendix 4) to obtain a final test concentration in a decreasing order from 80% to 16.67% elutriate.

2.5.2 Algae Growth Inhibition Test (AGI)

2.5.2.1 Test organism and culture preparation

The algae *Pseudokirchneriella subcapitata* culture was obtained from SAG-The Culture Collection of Algae of Göttingen University Germany (strain number 61.81). Algae pre-culture was prepared according to DIN EN ISO 8692 (2004). The original algal strains were inoculated in the nutrient rich KL-medium and left to grow for three months in the dark: light circadian cycles with a range of illumination between 70 and 87 µEinstein/(m^2s) (DIN 8692, 2012). This stock culture was used to inoculate new test cultures and incubated in the DIN medium of pH 8.1 ± 0.2 according to OECD

(1984) as shown in Fig. 2.2. Exponentially growing algae cells (1X10^{4}cells/mL) were exposed to different dilutions of sample elutriates.

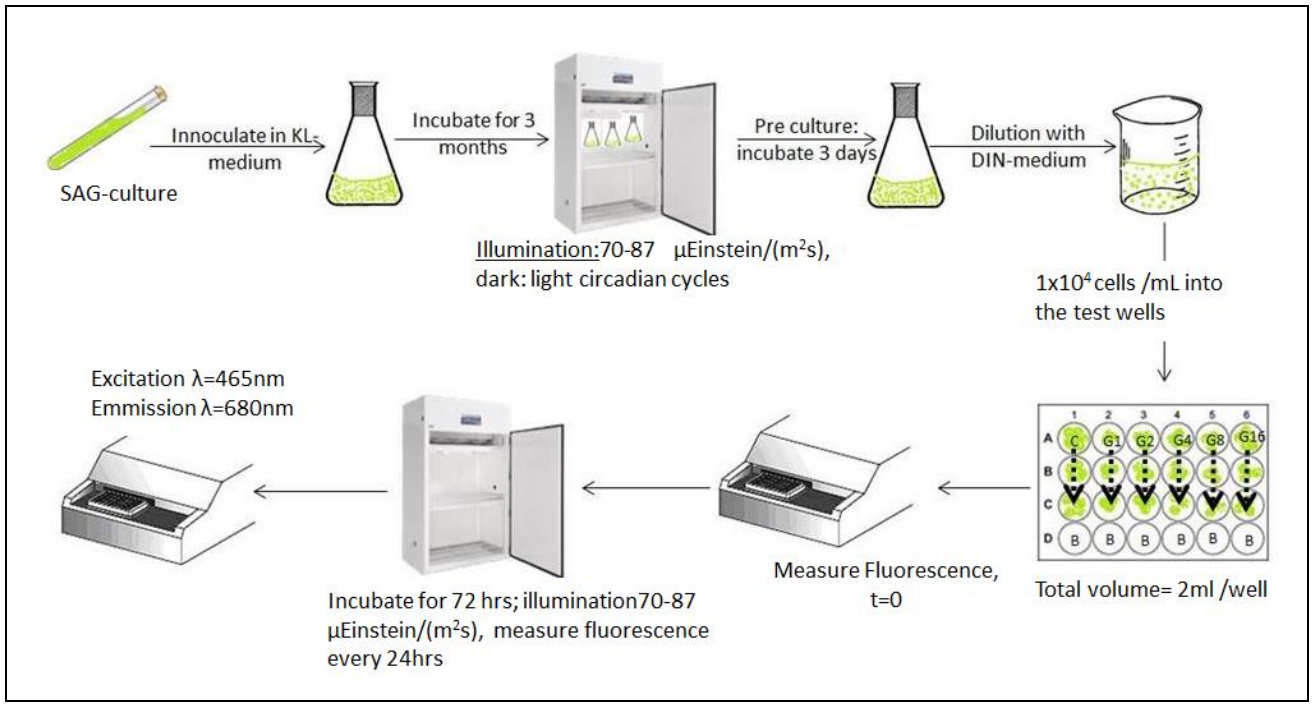


Figure 2.2: Schematic methodological procedures for P. subcapitata bioassay, C=control, B= Blank, G1-G16 = sample dilution steps

2.5.2.2 Experimental conditions

Three replicates of each test concentration or dilution step were used in a miniaturized 24 well cell culture plates. 200 µL of exponentially growing *P. subcapitata,* incubated under the conditions of the test for three days were added in to the well plates that contain the diluted samples and a nutrient rich medium (see Appendix 2-composition of the algal growth medium). The inoculated test plates were incubated in a continuous light in a range between 70 and 87 µEinstein/(m^{2}s) at 20 ± 2°C while shaking at 250 rpm. The auto-fluorescence emitted by chlorophyll was measured every 24 hours and the growth rate was calculated over 72 hours incubation time. Bi-distilled water was taken as negative control and freshly prepared solution of 10 mg/L of 3,5-dichlorophenol was used as positive control to verify the sensitivity of laboratory culture. Three replicate samples and controls were used (Fig. 2.2). Blank wells were used to correct any potential interference from the sample matrices, thus their values were deducted from the test or control values. The percentage inhibition or stimulation of the algae exposed to the samples was compared to that of reference substance and the controls.

2.5.3 Luminescent Bacteria Test (LBT)

2.5.3.1 Test organism and culture preparation

Bioluminescence inhibition of marine bacterium, *Vibrio fischeri* according to DIN EN ISO 11348-3 (2008) was used for water and soil or sediment elutriates. The freeze dried bacterium charge numbers 11229, 12164, 13037 and bacterial reconstitution activator solution were obtained from Hach Lange GmbH-Germany. 50 µL activated bacteria in ASW were exposed to 200 µL elutriates for 30 minutes at 15°C. ASW and 3,5-dichlorophenol (10 mg/L) were used as negative and positive controls respectively. Two replicate of each sample dilution step and controls were carried out. The bacteria luminescence was measured by using multimode micro well plate reader (TECAN Infinite-200) before and after the exposure to elutriate. Growth rate over 30 minutes was calculated as an endpoint response.

2.5.4 Bacteria Contact Assay

Bacterial Contact Assay (BCA) using *Arthrobacter globiformis,* a common soil bacterium which has an affinity to surfaces, was chosen in order to evaluate the toxicity of particle-bound contaminants according to DIN 38412-48 (2002). The test allows a direct eco-toxicological assessment of contaminated sediment or soil by measuring reduction of dehydrogenase activity because of inhibitory effect of contaminants. The bacteria are directly incubated with the sediments slurries, and the dye resazurin is converted, in the presence of the bacterial enzyme dehydrogenase, into resorufin, the concentration of which is measured by fluorimeter. The results are available within a day.

2.5.4.1 Test organisms and culture preparation

Bacteria culture preparation: The culture of *A. globiformis* (strain number 20124) was obtained from German collection of microorganisms and cell cultures, DSMZ. An overnight pre-culture of *A. globiformis* in 1/3 DSM medium (Appendix 3) was incubated for 14 - 16 hours in 30°C, with orbital shaking at 160 rpm for bacteria to acclimatize in a liquid medium. Then the following morning 1 mL of the pre-culture was transferred into 50 mL 1/3 DSM medium and incubated at the same conditions until the exponential growth rate of optic density of 0.3 was attained.

2.5.4.2 Sample preparation

10 g sediments or soil samples were first homogenized and aerated with 10 mL deionized water in a dark overhead shaker at 70 rpm for 48 hours. Sample slurries were pasteurized in a water bath at

80°C for 10 minutes to inactivate local soil microorganisms, and cooled in ice flakes for 10 minutes before measuring pH and redox potential. Sample slurries were diluted into four steps using deionized water in quartz sand in proportion to a respective dry weight of each sample. For soil samples, the proportion of quartz sand used for sample dilution were first adjusted to 50% of solid sample wet weight before adding deionized water in the same proportion to result into a total volume of 10 mL. A miniaturized method using 96 wells cell culture plates was employed. 120 µL of each sample dilution step of sediment slurries were pipetted into the test and calibration plates (Fig. 2.3).

2.5.4.3 Test procedure

40 µL of exponentially growing *A. globiformis* (O.D of 0.3) were added to all test wells and 1/3DSM medium to the blank wells. The micro-well plates were incubated for 2 hours in the dark at 30°C while shaking at 250 rpm. Quartz sand with 50% water content was used as reference sediment and quartz sand containing 20 mg/kg BAC (Benzyl-dimethyl-hexadecyl-ammonium chloride) was used as positive control. After 2 hours of incubation, 80 µL of redox indicating dye resazurin (45 mg/L in 0.1M MOPs buffer (pH= 8.2) was added and the reduced fluorescent dye product resorufin was measured using a TECAN-infinite 200 multimode plate reader every 15 minutes up to 45 minutes. Three replicates of each sample and control dilution steps were used. The calibrated slope of dehydrogenase kinetic between 15 and 45 minutes was used to calculate the % inhibition of dehydrogenase activity using the following equation:

$$\%\ Inhibition\ = 100 - \frac{\boldsymbol{S}dA\ sample}{\boldsymbol{S}dA\ control} \times 100\%$$

Where: $\boldsymbol{S} = Slope$ and $dA = dehydrogenase\ activity$

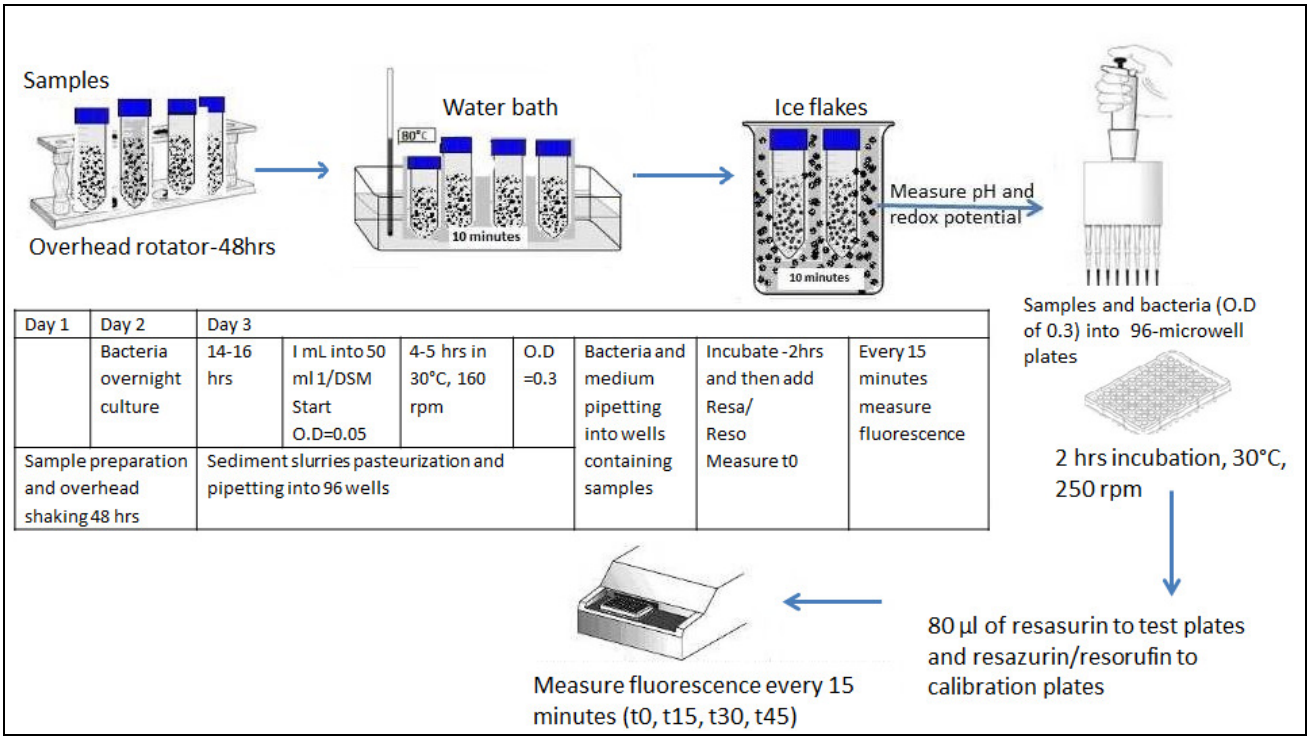


Figure 2.3: Schematic methodological procedures for A. globiformis culture and sample preparation before measuring fluorescence.

2.5.5 Acute Algae Test

2.5.5.1 Test chemicals:

Four chemicals were tested, i.e. two phenyl urea herbicides, diuron and isoproturon which inhibit electron transport system in photosystem II, glyphosate a herbicide which inhibit aromatic amino acids synthesis and 3,5-dichlorophenol (DCP) which inhibit oxidative phosphorylation in plant respiratory system. All pesticides were purchased from Sigma-Aldrich Chemie GmbH, Germany. Dimethyl sulfoxide (DMSO) 99.5% pure was supplied by Carl Roth GmbH & Co.KG, Germany and 3,5-dichlorophenol (DCP) was supplied by Alfa Aesar GmbH & Co KG, Germany. Seven different end concentrations of the test chemicals were used. For isoproturon, diuron and 3,5-DCP concentrations were; 0.035, 0.105, 0.35, 1.05, 3.5, 10.5 and 35 µg/L, while for glyphosate concentrations were; 0.6, 0.7, 0.84, 1.05, 1.4, 2.1 and 4.2 mg/L. 3,5-DCP and glyphosate were dissolved in bi-distilled water while diuron and isoproturon were dissolved in DMSO in which the final concentration in the wells was maintained less than 1% DMSO.

2.5.5.2 Algae pre-culture and optimization

The algae *P. subcapitata* (strain number 61.81) was obtained from SAG -Algae Culture Collection Centre- Göttingen, Germany. The algae was cultured and incubated with DIN-mediums according to Germany Industrial Standards DIN EN ISO 8692 (2004). Cell density of an exponentially growing (a 3-day pre-culture) algae prepared according to (OECD, 1984) were measured (in terms of fluorescence) in both 24 and 96 micro well plates by using fluorimeter, TECAN-Infinite 200-Multimode micro-plate reader, to assess its growth and fluorescence capacity in the two plates. Both prompt fluorescence (PF) and delayed fluorescence (DF) were measured every after 30 minutes up to 120 minutes, leaving a 5 minutes interval between the PF and DF while incubating the algae in a continuous light in a range of 70 and 87 µEinstein/(m^2s) at 20 ± 2°C, 250 rpm. Integration time and lag time were varied in DF to obtain ideal conditions for fluorescence units detection using multimode microtiter plate reader. Depending on the results of these experiments four test conditions for delayed fluorescence measurement were chosen: (i) 30 minutes incubation of algae cells with test chemicals (ii) algae cell density of 2x10^4cells/mL (iii) integration time of 80 microseconds and (iv) lag time of 100 microseconds. In prompt fluorescence both integration and lag time were zero. The excitation and emission wavelengths for both fluorescence measurements were 465 nm and 680 nm respectively. The excitation and emission bandwidths were set at 35 nm and 30 nm respectively. In order to ensure best results for fluorescence measurements, optimal gain up to 200 for delayed fluorescence and 53 (manually set) for prompt emissions were used.

2.5.5.3 Experimental set up

96-flat bottomed micro well plates were used in this study in order to assure maximum concentration of algae within short time in a small volume for optimal detection of delayed and prompt fluorescence. 100 µL of exponentially growing algae were exposed to 100 µL of each of the seven concentrations of the test chemicals in a nutrient rich medium in batch cultures for 30 minutes. Three replicate wells for each concentration of the test chemical and the control were used. Wells had same nutritive medium, experimental conditions and algae cells from same pre-culture except for blanks where 100 µL of 1*X* concentration DIN medium was added instead of algae. 10 µL of 10*X* concentration DIN medium was added in all wells in order to provide enough nutrients for algal growth. Bi-distilled water in 1% DMSO was used as a control for diuron and isoproturon test plates, while for 3,5-dichlorophenol and glyphosate plates bi-distilled water was

used as a control. Test plates were incubated for 30 minutes in a continuous illumination from fluorescent lamps of 70 and 87 µEinstein/(m^2s) at 20 ± 2 °C in an orbital shaking at 250 rpm.

In the second experimental set up, aforementioned concentrations of the four test chemicals (Section 2.5.5.1) were added to samples, which showed extreme results of either low or high percentage inhibition of growth rate in the conventional 72 hours algae test. Algae cells were exposed to spiked sample elutriate, incubated for 30 minutes and afterwards both DF and PF were measured.

2.5.5.4 Cell density in relation to fluorescence measurement

In AGI experiments final algae cells density after exposure to test chemicals were determined by counting the cells with an inversed microscope at bright field illumination. Counting was conducted using Neubauer-improved chamber or hemocytometer at 400x total magnification as described by Oscar Bastidas (www.celeromics.com). Four diagonal counting grids were counted for each of two sides of sample detection or cell counting area (Fig. 2.4). To reduce bias, cells touching the middle line on the bottom and left of the counting grid were included while those touching the middle line on the top and right were excluded.

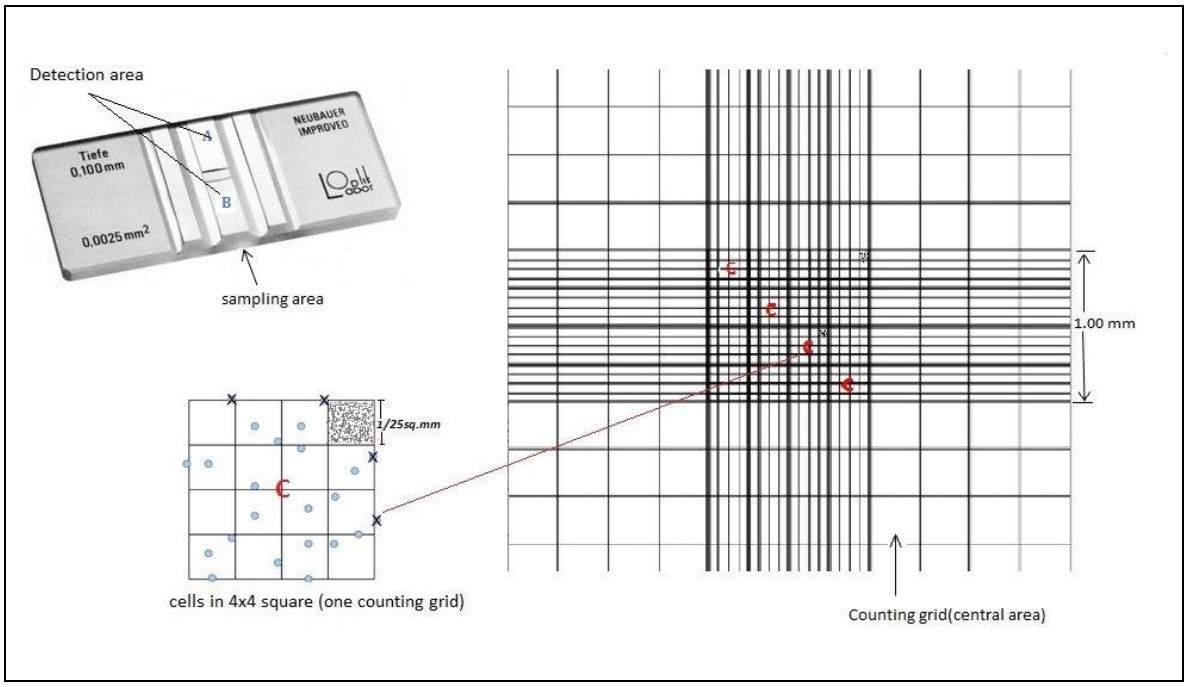


*Figure 2.4: Sketch of algae cell counting technique in an Improved Neubauer Chamber. The red letters "**C**" in the middle of big square indicates four diagonal grids that were considered during counting.(modified from www.celeromics.com)*

The average number of cells was calculated from the two counting areas of the hemocytometer. Total cell concentration per well was calculated from the number of cells counted and the total volume of the counted grids as shown in the following equation:

- $Area\ of\ one\ big\ square = 1mm \times 1mm = 1mm^2$
- $Volume\ of\ one\ big\ sguare = area \times depth\ of\ cover\ slip\ (0.1\ mm)$
  - $1mm^2 \times 0.1\ mm = 0.1mm^3$which is equivalent to$1 \times 10^{-4}mL$

$$Cell\ density\ (cells/mL) = \frac{Total\ cells\ counted}{Number\ of\ squares} \times 10{,}000$$

Fluorescence per cell was determined in each dilution step of the test chemical by dividing the measured auto-fluorescence by the cell density of a specific well.

2.5.6 Yeast Resazurin Test Assays that Measure Metabolic Capacity

This test was conducted according to Fai and Grant (2009b), with some modifications to suit the conditions and equipment of our laboratory.

2.5.6.1 Principle of the test

S. cerevisiae reduces resazurin to resorufin, thus the fluorescence measurement indicates the contaminant dose response to resazurin reduction. Inhibition of resazurin reduction indicates an impairment of cellular metabolism. Conversion of resazurin to resorufin by viable cells results in fluorescence, which is proportional to the number of viable cells (Fig. 2.5).

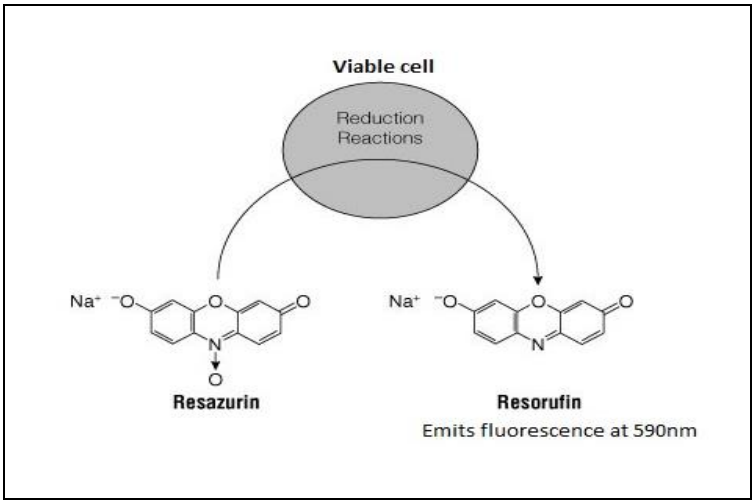


Figure 2.5: Resazurin conversion into resorufin by reduction reactions of metabolically active cells (www.promega.de)

2.5.6.2 Yeast strain, medium and culture conditions

The *S. cerevisiae*-strain NCYC 2939 used in this study was purchased from UK-National Collection of Yeast Cultures (NCYC – Institute of Food Research, Norwich Research Park, Norwich, UK), Amp. batch 09/07. The yeast cells were inoculated and cultured at 30°C for 48 hours in sterile 1% **y**east extract, 2% **p**eptone, 2% **d**extrose and 2% **a**gar (sterile YPD -agar plate) prepared from 1000 mL of deionized water. YPD medium which contained (1% **y**east, **P**eptone 2%, **d**extrose 2%) was prepared by dissolving 10 g yeast extracts, 20 g peptone, 20 g dextrose (glucose) in 1000 mL of deionized water. However, in order to promote optimal growth of yeast cells, darkening of the medium was avoided by autoclaving dextrose solution separately before mixing with the rest of autoclaved ingredients. Yeast culture was prepared by harvesting 1 to 2 colonies of the yeast cells into 50 mL YPD medium (pH 7.00) prepared according to (Treco & Lundblad, 2001) and incubated at 30°C, 250 rpm overnight. In the next morning, 500 µL of overnight culture were transferred into 50 mL YPD medium in an Erlenmeyer flask and incubated at 30°C, while shaking vigorously at 300 rpm. The O.D of the culture was monitored frequently until when $O.D_{600}$ of 0.5± 0.01 was reached, the yeast cells were introduced into the test plates. The ability of yeast cells to grow in liquid medium was optimized for efficient use in this study.

2.5.6.3 Test chemicals

Organic fungicides, prochloraz and thiabendazole PESTANAL ®analytical standard, hydrated copper (II) sulphate ($CuSO_{4.}5H_20$), resazurin dye, yeast extract, peptone, dextrose, agar and glucose were purchased from Sigma-Aldrich Chemie GmbH, Germany. Dimethyl sulfoxide (DMSO), 99.5% pure was supplied by Carl Roth GmbH & Co.KG, Germany. Phosphate buffered saline (1X concentration PBS) of adjusted pH value of 7.5 using 0.165 M 3-(N-morpholino) propanesulfonic acid (MOPS) was used to dissolve resazurin in order to maintain high pH for the dyes to remain in their anionic form for good fluorescence emission (Bueno *et al*., 2002). 0.1 M (10*X* concentration medium) of PBS stock solution was prepared in advance as a mixture of 10.9 g of $Na_2HPO_{4,}$ 3.68 g of $NaH_2PO_4.H_2O$ and 90 g of NaCl in 1000 mL of bi-distilled water. The buffer was autoclaved and stored at 4°C for use in all yeast tests.20 µL of resazurin (prepared as 11 mg resazurin/100 mL of 1X PBS of adjusted pH value of 7.5 by MOP) and 96 round bottomed transparent micro-wells plates were used. All organic solutions of fungicides concentrations were prepared in DMSO in which its concentration was maintained at 1%.

2.5.6.4 Resorufin fluorescence inhibition experiments

Preliminary tests were carried out to determine the growth rate of yeast and to establish yeast density, which could reduce resazurin at an appropriate length of incubation with test chemicals. *S. cerevisiae* cells were exposed to different concentrations of the test chemicals in varying length of time, starting from 1, 2, 3, 6 and 24 hours. Toxicant dilutions were prepared in the desired concentration and 100 µL introduced into the microplates wells. An equal volume, 100 µL of yeast suspension of $O.D_{600}$ =0.5± 0.01 was added into the control and test wells, except for blanks where YPD was introduced instead of yeasts. All test plates were covered with breathable seal paper, then incubated at 30°C for aforementioned length of periods with orbital shaking at 250 rpm. The control wells consisted of yeast cells and deionized water in 1% DMSO for organic fungicides and deionized water for copper (II) sulphate, which was not dissolved in DMSO. Lastly, after each incubation period, 20 µL of resazurin was added to all wells and incubated further for 40 minutes in 30°C, 250 rpm, before measuring fluorescence. Due to rapid reduction of resazurin by *S. cerevisiae*, all three replicates were conducted in parallel in the same type of plate at the same settings. This is because in the prior optimization experiments, substantial differences were detected in separate plates where readings were taken in 2 - 3 minutes apart. Therefore, a varied microplate configuration was used in which all three replicates for each test concentrations were on the same plate. Fluorescence was measured by Microplate reader, TECAN Infinite 200.

2.5.6.5 Exposure of *S. cerevisiae* to environmental samples

Dry season sediment, soil and water samples collected from KVRS were used to assess the viability of yeast metabolic activities when exposed to contaminated samples.

Sediment and soil elutriates were prepared exactly the same as explained in the AGI (refer Section 2.5.1). Sediment and soil elutriate were centrifuged for 20 minutes and 60 minutes respectively at 10,000 g in order to obtain a clear solution to optimize the growth rate of yeast cells. pH values of each sample were recorded before pipetting into the micro-well plates and three replicates of each dilution step were used in a miniaturized round transparent 96 well cell culture plate. 100 µL of samples and $CuSO_4$ were introduced into all test and control wells respectively. An equal volume, 100 µL of yeast, *S. cerevisiae* suspension of $O.D_{600}$ =0.5± 0.01, prepared according to Section 2.5.6.2 was added into the control and test wells, except for blanks where 100 µL of YPD was introduced instead of yeasts. All test plates were covered with breathable seal paper, incubated at 30°C for 4 hours with orbital shaking at 250 rpm. 20 µL of resazurin (prepared as 11 mg resazurin/100 mL of

1X PBS of adjusted pH value of 7.5 by MOP) was added to all wells. Initial fluorescence was measured, plates were further incubated for 40 minutes at 30°C, 250 rpm. Final fluorescence was measured at excitation and emission wavelength of 530 nm and 590 nm respectively. Bi-distilled water was used as negative control and seven different concentrations of $CuSO_4$ were used as positive control in the logarithmic range of 0.3, 1, 3, 10, 30,100, and 300 mg/L. The percentage inhibition of metabolic activity of yeast exposed to the samples was compared to those of negative controls.

2.6 Classification of results to integrate toxicity responses

Only sediment and soil samples were included in the classification system because they were subjected to the three bioassays (LBT, AGI and BCA). Water samples were subjected to only two bioassays (AGI and LBT), therefore they were excluded from the classification system.

2.6.1 Fuzzy Rule Based classifications

A wide range of *P. subcapitata, A. globiformis and V. fischeri* bioassay results data that have been gathered and stored in the database of the Applied Aquatic Toxicology Research group of Hamburg University of Applied Science, Faculty of Life Sciences were used to derive the toxicity classes. The bioassay, database consisted of more than 200 sediment and water samples collected from Elbe River in Germany from 2009 to 2014. These data were obtained by the same methods as those applied to sediment and soil samples of KVRS. Besides, the data contained a wide range of bioassay toxicity responses, which varied from very low to extreme inhibition responses due to historic contaminations and other up-stream anthropogenic activities. Toxicity responses were calculated as the percentage of inhibition resulted by samples compared to negative controls. Box whiskers plots with median, 25, 75 percentiles (Fig. 2.6) were used to get an overview of the specific potential response span of different bioassays and in what manner data were distributed within the range of a wide database results with different environmental risks. The box plots were used to derive the range of the toxicity categories.

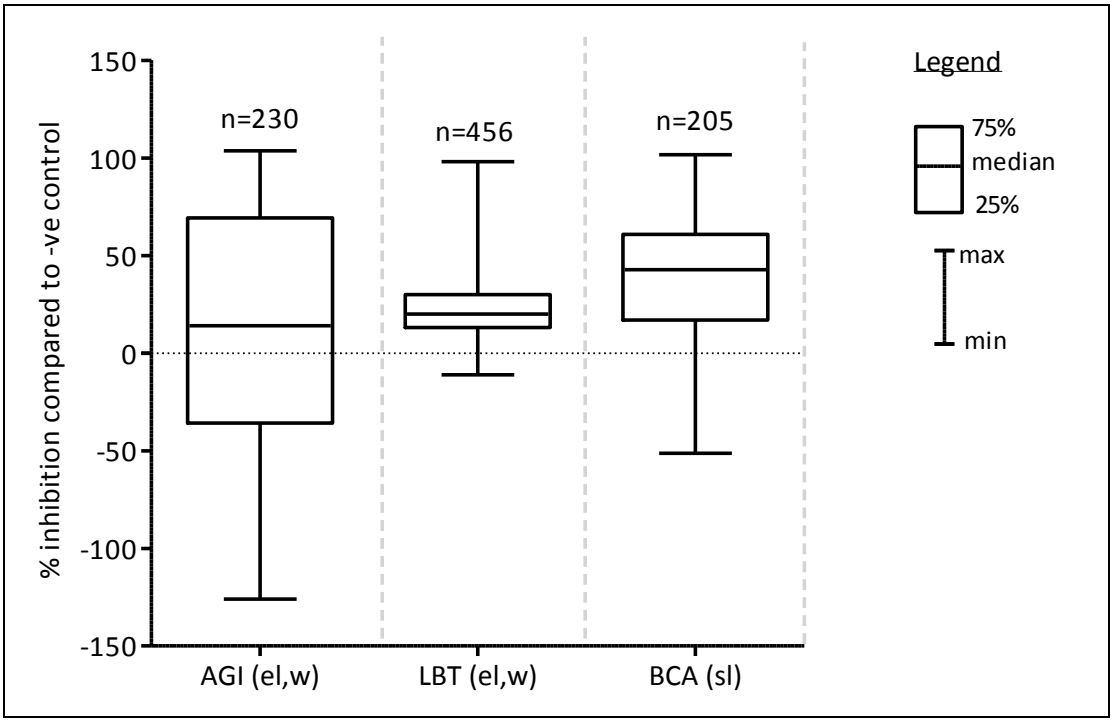


Figure.2.6. Box whisker plots of response of 3 bioassays results to the respective matrix (el=elutriate, w=water, sl=sediment slurries) derived from n-samples. AGI=Algae growth inhibition test, LBT =Luminescence bacteria test, BCA =Bacteria contact assay with sediment slurries.

2.6.2 Toxicity categorization and fuzzy classes

Three categories of toxicity range among the wide responses of sediment samples were possible due to potential overlap from the standard deviation among the replicates of the tests. Hence, Category 1: “Little or no response”, ranged from the lowest extreme response to the 25 percentile. Category 2: “Moderate response” comprised of responses that fall between the lower 25 and higher 75 percentile. In this category, more than 50% of sediment data were located. Category 3: “high response” consisted of all elevated responses from higher 75 percentile to maximum values, in this case up to 100% inhibition. Table 2.1 summarizes the range of each category for each bioassay inhibition response, deduced from the box whisker plots according to Ahlf and Heise (2005).

Table 2.1: Assignment of biotests results to fuzzy sets. The ranges of the responses were deduced from the results of each bioassay

Toxicity category	AGI (n=230)	LBT (n=456)	BCA (n=205)
Little or no response	<35%	<15%	<17%
Moderate response	35-70%	15-30%	17-60%
High response	>70%	>30%	>60%

Average standard deviations of the mean inhibition among the replicates of positive and negative controls were used to indicate specific overlap values, which also define the fuzzy slopes. The variation among replicates of positive controls indicates the precision of the test procedures and the influence of sample matrices to test organisms. In order to determine the eco-toxicological risk assessment of Kilombero valley, results from the three standardized bioassays of each sediment sample were used to estimate the overall toxicity response in comparison to the derived categories from the wide range of the database data. The combination of fuzzy sets within the rule base was calculated according to Ahlf and Heise (2005) by using the following inferences, (i) operator of aggregation: minimum, (ii) operators of implication; algebraic product, (iii) operator of accumulation: maximum. Inferences of the rules application were drawn by *"if….and….then…."* rules for each sample according to responses in the three biotests. For instance;

- **Rule 1**: *If* the toxicity category of AGI is "high response" *and* BCA is "moderate response" *and* LBT is "high response", Conclusion: *then* Classification is "elevated critical risk".
- **Rule 2.***If* the toxicity category of AGI is "little or no response" *and* BCA is "little or no response", *and* LBT is "moderate" response, Conclusion: *then* Classification is "little or no potential risk".

The list of all fuzzy rules used in this study is presented in Appendix 5.

Total contribution of the three bioassays/ variable to a rule weight was equal to 1. MIT Data engine software Package-Version 4 was used in this classification system.

2.7 Data and Statistical analysis

All statistical analysis and graphs were prepared using a Graph Pad Prism5 statistical package. One-way analysis of variance (ANOVA) was used to compare seasonal variation among the bioassay results. In AAT the same was used to compare the inhibition or stimulation values among the four pesticides in both PF and DF in repeated measures of the matched test concentrations.

For Yeast test, percentage resorufin fluorescent inhibition was calculated by Microsoft office- Excel using the mean values in control and the replicates of the treatment groups. Data reported are mean values of at least five independent resorufin fluorescence inhibition experiments carried out under identical conditions, as indicated in the figures legend. One way ANOVA was used to compare the differences in percentage of resorufin inhibition or stimulation among the three fungicides in different incubation periods and in different test concentrations. The responses of *S. cerevisiae* to different incubation time with fungicides was compared by Tukey's Multiple Comparison Test. Paired t-test was used to compare the differences in resorufin fluorescence production with the control groups in each test concentration.

Elemental associations among heavy metals and major elements in the soils and sediment samples were investigated by using Pearson correlation matrix. Factor Analysis using Principal Component Analysis (PCA) was used to find a correlation between bioassays results and the analytical parameters such as C/N ratio, metals and grain size parameters of sediment and soil samples. Extraction parameters were: Minimum eigenvalue, 1, Convergence: 0.001 and 25 interactions with 3 factor components. Principal Component Analysis was performed by MYSTAT 12 for windows - Version 12.02.00 (A Student Version of SYSTAT).

CHAPTER THREE: RESULTS

3.1 Analytical results

In order to integrate the toxicity response of the bioassay results into a risk assessment, analytical parameters such as sample C/N ratios, grain size, trace and heavy metals screening, pesticide screening and other geo-physical parameters of collected samples were carried out.

3.1.1 Kilombero samples C/N ratio analysis

Total carbon and nitrogen in the sediment and soil samples were significantly correlated (r^2= >0.9, p<0.0001, Fig. 3.1). Soil samples had lower total percentage of C and thus wider C/N ratios than sediment samples (Table 3.1). The overview range values of each parameter measured in the field and laboratory are summarized in Table 3.2. Only one sediment sample had a wide C/N ratio of 22.1. The rest of sediment and soil samples had narrow C/N ratios less than 20.

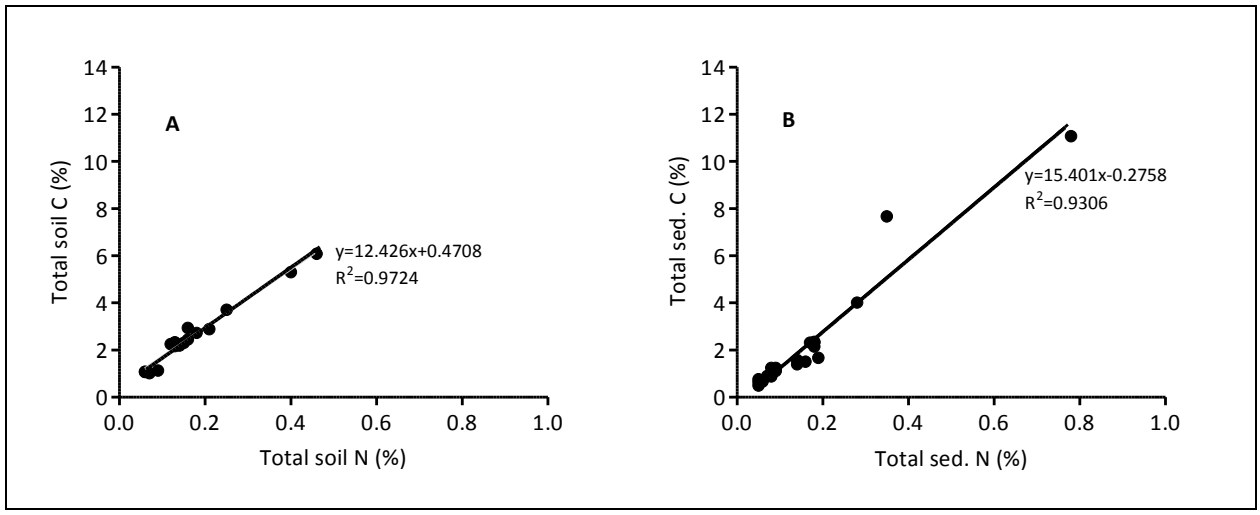


Figure 3.1: Total percentage of carbon and nitrogen for the (A) soil samples (n= 17) and (B) sediment samples (n=22) collected in Kilombero Valley Ramsar site

3.1.2 pH, salinity and electrical conductivity of collected soil and sediment samples

Of all 38 samples (sediments and soils) that were measured, only one sample, IP-1 from Idete prison, showed a neutral pH, while the rest of soil and sediment samples from all sampling stations were either slightly or acidic in nature (Table 3.1). Soil electrical conductivity (EC) varied from one sampling station to another. Soil EC is a measure of soil salinity, and an indicator of the quantity of available nutrients in the soil, available for crops to absorb.

Table 3.1: Summary of the range of specific units of measured physical parameters of 38 samples (sediments and soils) from each sampling station, na= not available.

Station-sample	C/N ratio	% Salinity	Electrical conductivity (µS/cm)	pH	Estimated grain size fraction (% DW)	
					>63 µm (fine sand)	<63 µm (silt + clay)
[a]IP1	13.12	0.05	103.30	7.14	30.30	69.90
[a]IP2	14.30	0.03	49.50	5.85	-	-
[a]IP3	15.86	0.02	-	5.32	11.30	88.90
[a]IP4	14.08	0.02	39.77	5.31	26.80	73.20
[b]IP9	15.06	0.02	41.23	4.87	8.60	91.50
[b]IP10	13.31	0.02	55.01	4.97	-	-
[b]IP11	13.89	0.03	34.76	5.23	30.80	69.30
[b]KP1	19.38	0.02	57.89	4.77	-	-
[b]KP2	17.93	0.04	-	5.79	36.70	63.40
[b]KP3	15.86	0.03	49.87	4.93	8.90	91.20
[b]KP4	17.52	0.03	43.95	5.04	-	-
[a]KPL1	14.29	0.02	-	5.34	-	-
[a]KPL2	13.99	0.02	39.17	5.26	63.70	36.40
[a]KPL3	13.59	0.02	-	5.77	33.40	66.70
[a]KPL4	13.22	0.05	102.07	6.58	68.80	31.30
[a]KPL5	12.18	0.04	67.73	6.72	-	-
[a1]KPL6	11.39	0.02	41.70	5.27	18.60	81.40
[b]KPL19	13.16	0.02	50.40	5.17	27.90	72.20
[a]KSC1	13.19	0.04	50.00	6.47	62.10	38.00
[a]KSC2	9.44	0.05	90.97	6.97	28.50	71.40
[a]KSC3	11.70	0.03	59.43	5.10	-	-
[a]KSC4	10.26	0.04	-	6.58	-	-
[a]KSC5	11.04	0.04	-	6.72	31.70	68.30
[a]KSC6	11.91	0.02	69.23	5.61	76.20	23.90
[a]KSC7	11.10	0.04	-	5.79	-	-
[a]KSC8	8.95	0.05	74.43	6.17	14.10	85.90
[a]KSC9	22.10	0.03	-	5.10	20.00	80.10
[a]MBS1	12.11	0.03	45.33	6.88	20.90	79.10
[a]MBS2	13.02	0.03	53.73	5.86	62.50	37.60
[b]MBS6	15.85	0.02	-	5.49	-	-
[b]MBS7	18.95	0.02	38.83	5.29	49.5	50.7
[b]MBS8	15.43	0.02	-	4.77	-	-
[b]MBS9	17.84	0.02	-	5.76	15.00	85.00
[b]MBS10	17.66	0.03	45.47	5.24	-	-
[b]TAC5	15.06	0.02	39.23	5.34	19.5	80.6
[b]TAC6	16.37	0.02	35.03	4.87	-	-
[b]TAC7	15.10	0.02	39.67	4.72	14.4	85.8
[b]TAC8	13.34	0.03	56.30	4.89		

[a]sediment, [b]soil

Table 3.2: Overview of the range values of measured physical-chemical parameters of 22-sediment, 17-soil and 27-water samples. na= not available.

Sample matrix	Measured on site		Laboratory measurement				
	Temp. (°C)	pH	pH	EC (µS/cm)	Salinity(%)	C:N ratio	% DW
Sed.	24.2-38.7	5.07-7.80	5.10-8.05	39.17-103.30	0.01-0.04	8.95-22.09	22.1-66.3
soil	na	Na	4.72-5.79	34.76-57.89	0.02-0.04	13.17-19.38	53.5-81.4
water	24.3-40.1	4.95-8.64	6.71-8.05	Na	0.01-0.06*	Na	na

**Salinity of water samples was measured by using a refractometer*

An overview of worldwide soil properties classification, according to measured range of physical parameters is presented in Chapter 4, Table 4.1. The class limit or boundaries may change depending on the intended use of the data (FAO, 1983, Sys *et al* 1993). Therefore, the classes presented in this study are generalized according to spatial database of soil depth of >20 to 100 cm, from WISE-DSMW *(World Inventory of Soil Emission Potentials FAO -UNESCO Soil Map of the World).* Data used in this classification system, were obtained from various soil laboratories, over a range of years, using various analytical methods established by International Soil Reference and Information Centre (ISRIC) (Batjes, 2012)

3.1.3 Dry weight and grain size analysis

Grain size fractions of sediment samples within a single station were in the same magnitude as soil samples. However, there were a significant differences between the grain size fractions among 51 measured samples *($p<0.05$, 1-way ANOVA, post-hoc, Dunn´s Multiple comparison test)* (Fig. 3.2). The finest particle size fraction (<20 µm) was predominant in all samples. However, 61% and 69% of sediments (n=34) and soil (n=17) samples, respectively, had a silt-clay texture (<0.1 mm), while 39% and 31% of sediment and soil samples, respectively, were sandy-clay (0.10 mm to 2 mm).

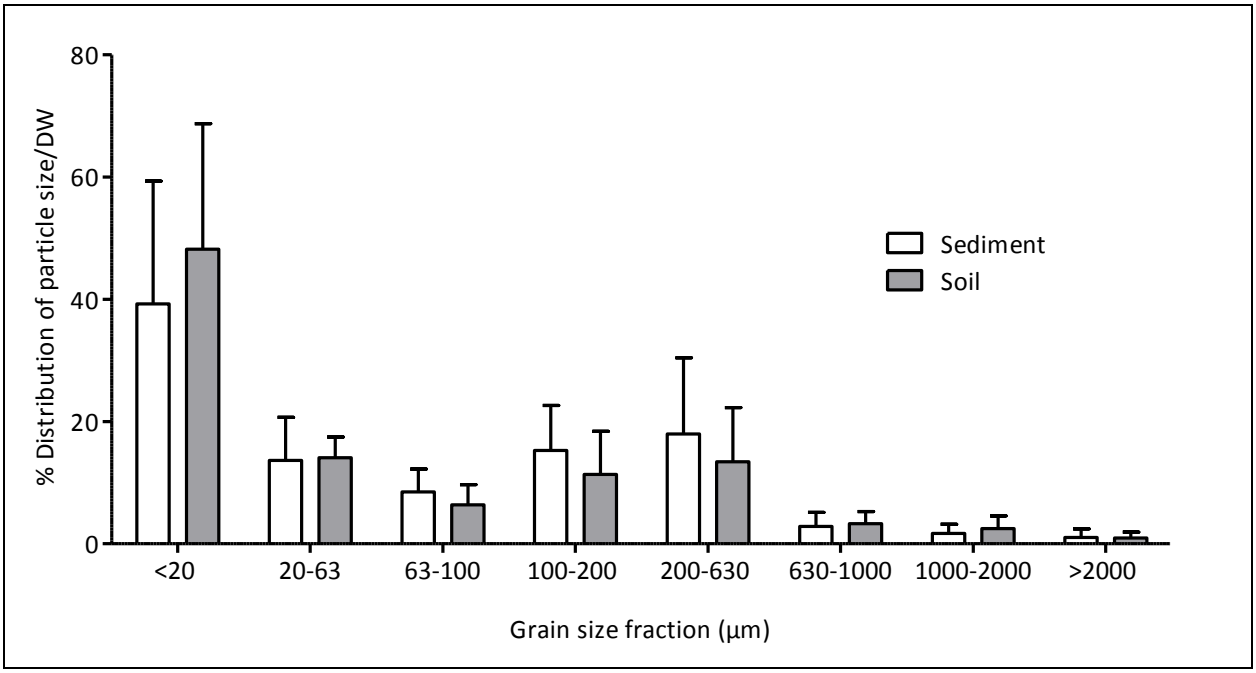


Figure 3.2: Average percentage distribution of grain size fractions per dry weight of sediment (n=34) and soil (n= 17) samples collected in various sampling stations of Kilombero Valley.

3.1.4 Pesticide screening results

Table 3.3: Minimum, maximum and mean concentration of detected pesticides in sediment, water and soil samples. Concentration values are expressed as µg/L (water) and µg/g DW (soil and sediments) respectively.

Pesticides	Matrix	Rainy season-2012			Freq. of detection per (n-samples)	Dry season-2013			Freq. of detection
		Concentration				Concentration			
		min	max	mean		min	max	mean	
1.Organophosphorus									
chlorpyrifos	SED	0.12	1.83	0.975	3(12)	0.045	0.092	0.0685	2(20)
glyphosate	Water	-	-	nd		0.035	0.05		2(13)
	Soil	Na	-	-		0.01	0.55	0.15	5(17)
	SED	-	-	0.24	1(12)	0.043	0.19	0.1043	3(20)
AMPA	Soil	Na	-	-		0.01	0.49	0.18	3(17)
2.Triazines									
atrazine	Water	0.11	0.52	0.315	2(8)	nd	nd	-	-
	SED	0.645	0.56	0.73	2(12)	-	-	0.46	1 (20)
2-hydroxy-atrazine	Water	0.095	0.11	0.1025	2(8)	nd	-	-	-
	SED	0.56	0.73	0.645	2(8)	nd	-	-	-
3.Triazinone									
hexazinone	Water	-	-	0.097	1(8)	nd	-	-	-
metribuzin	Water	0.12	0.182	0.151	2(8)	nd	-	-	-
4. *Phenyl urea*									
diuron	SED	-	-	0.16	1(12)	0.026	1.1	0.236	6(20)
	Water	0.053	1.7	0.651	3(8)	nd	nd	-	-

	SOIL	Na	na			-	-	0.052	1(17)
monuron	Water	1.28	3.9	2.59	2(8)	nd	-	-	
desmethyl diuron	SED	0.053	0.11	0.082	2(12)	0.05	0.096	0.073	2(20)
5.Carbamate									
propoxur	Water	0.055	0.083	0.067	5(8)	nd	-	-	
	SED	0.055	0.067	0.061	2(12)	nd	-	-	-

na= not available for particular parameter, nd=not detected

Type of pesticide, its concentration and frequencies of detection varied according to season and sample matrices. Of more than 60 pesticides which were screened for, diuron, glyphosate, and propoxur were frequently detected (Table 3.3). There was more detection of pesticides residuals in rainy season than dry season water samples. However, a number of pesticides were detected in both rainy and dry season sediment and soil samples. For instance, chlorpyrifos was detected in both dry and rainy seasons (in sediments only), glyphosate and AMPA were more detected in dry season (in sediment and soils), atrazine and 2-hydroxyatrazin were more frequently detected during rainy season (in sediment, soil and water) samples. Phenylurea herbicides were detected in both dry and rainy seasons in sediments, water and in soils. Overall summary of detected pesticides per sampling station is presented in Table 3.4. Diuron was frequently detected in sugarcane plantations, solely or in combination with other pesticides in the water or sediment samples.

Table 3.4: Major crop types in relation to type of pesticide detected in the sampling plantations

Sampling station	Pesticide detected	Environmental compartment	season	Major crop types
Chita Ns	ND	-	-	paddy rice
IP	glyphosate, AMPA	sediment, soils	dry season	paddy rice
KP	Diuron	Soil	dry season	sugarcane
KPL	Chlorpyrifos	sediment	rainy	paddy rice
KSC	AMPA, diuron, desmethyldiuron, 2-hydroxyatrazin, propoxur, hexazinone, monuron, metribuzin, atrazine	sediment, soil and water	rainy, dry	Sugarcane
Teak Co.	ND	-	-	Teak
MBS	2-hydroxyatrazin	water	Rainy	paddy rice
TAC	ND	-	-	paddy rice

ND= no pesticides were detected

3.1.5 Elemental and heavy metal screening

Table 3.5: XRFA results of mean metal concentration (mg/kg DW) and % recoveries of selected metals from the certified standard reference material SRM ®2711a

Metal	Symbol	Measured conc. (mg/kg	Certified conc. (mg/kg)	% Recovery
zinc	Zn	358.7 ± 9	414 ± 11	86.63
cadmium	Cd	53.7 ± 10	54.1 ± 0.5	99.20
copper	Cu	124.3 ± 7	140 ±2	88.81
lead	Pb	1352.7 ± 17	1400 ±0.001	96.62
arsenic	As	99.3 ± 10	107 ± 5	92.83
titanium	Ti	2312.7 ± 135.3	3170 ± 0.008	72.95
vanadium	V	72.3 ± 14.4	80.7 ± 5.7	89.63
manganese	Mn	513.3 ± 27	675 ± 18	76.05
iron	Fe	23424.7 ± 221.7	28200 ± 0.04	83.07
rubidium	Rb	117.3 ± 3	120 ± 3	97.78
strontium	Sr	238.3 ± 7.3	242 ±10	98.48

SRM=certified standard reference material, Conc = concentration

Percentage recovery of reference material from the certified concentrations of SRM®2711a varied among metals (Table 3.5). However, the measured XRFA metal concentrations were essentially in the same magnitude as the certified values. Therefore, the results obtained from environmental samples were considered acceptable and reliable for the purpose of this study.

Figure 3.3 indicates mean concentration of the detected metals and extreme values in the lower and higher 25% and 75% percentiles, respectively. There was a large variation between the maximum and minimum concentrations of various elements in the sediments and soils samples. Table 3.6 provides an overview of mean and standard deviations of metals concentration for each sampling station. When comparing metal concentration among samples, KPL showed elevated mean concentrations of Mn and Cr while MBS had lower mean concentrations of Mn, Sr, Fe and Zn. Moreover, Zr concentration was highly elevated in one sample from TAC with a concentration of 1165 mg/kg DW. This indicates the extent of background metal variations in the sampling stations. Apart from Fe and Mn, which are major components of sediments, concentrations of Cr and Zr were highly elevated in most of the samples compared to other metals, with an average concentration of 134.9 mg/kg DW and 445.2 mg/kg DW, respectively. Nickel was detected only in 6 out of 41 sediment samples, with concentration between30.0 mg/kg and 77 mg/kg DW.

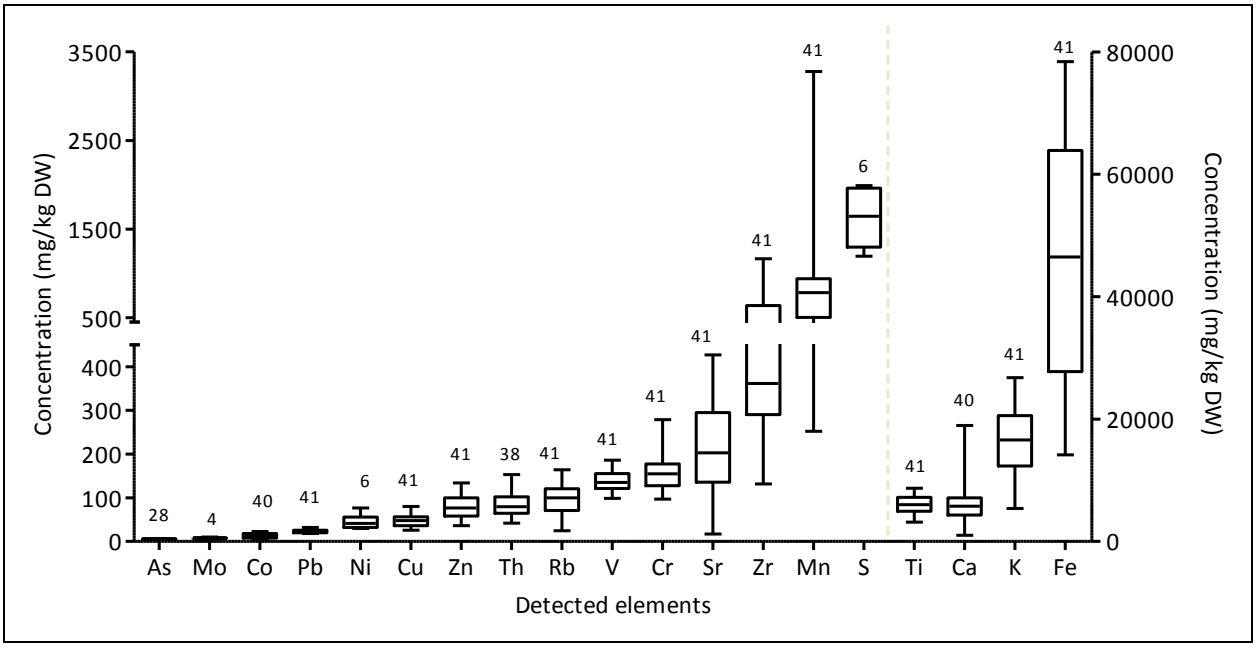


Figure 3.3: Box whiskers plots showing minimum, maximum and mean concentrations (mg/kg DW) at 95% confidence interval of metals and other elements detected in sediment and soil samples of Kilombero valley. The numerals on top of the bars represent frequency of detection of a particular element, n= 41. Right Y-axis consists of Ti, Ca, K and Fe.

Table 3.6: Overview of metal concentrations (expressed as mean ± standard deviation in mg/kg on dry weight basis) of detected metals for each sampling stations

METALS	Sampling stations					
	TAC (n= 5)	MBS (n=8)	IP (n=7)	KP (n=4)	KSC (n=11)	KPL (n=7)
As	5.3 ± 0.8	5.7 ± 0.7	5.6 ± 0.7	4.8[1]	5.7 ± 0.7	5.8 ± 0.5
Co	14.4 ± 7.0	12.0 ± 6.6	11.1 ± 6.6	11.1 ± 6.6	13.1 ± 4.9	14.0 ± 5.9
Cr	170.5 ± 30.9	156.9± 43.2	151.7 ± 32.4	149 ± 32.1	152.1 ± 38.9	**167.0 ± 60.1**
Cu	50.8 ± 13.2	41.4 ± 17.9	42.3 ±9.6	38.3 ± 7.4	54,0 ± 15,9	46.6 ± 13.4
Fe[a]	**51.5 ± 29.9**	40.6 ± 23.1	36.6 ± 19.6	44.0 ± 17.7	46.3 ± 18.9	53.9 ± 18.9
Mn	879.3 ± 410.5	603.0 ± 286.1	628.6 ± 352.5	623 ± 300.4	721.9 ± 258.3	**1236.1 ± 919.9**
Ni	ND	44.0[1]	39.0[1]	31.5 ± 2.1	49.0[1]	77.0[1]
Pb	23.5 ± 1.7	22.6 ± 7.2	22.3 ± 3.4	21.0 ± 3.5	25.0 ± 4.9	24.6 ± 2,5
Sr	229.3 ± 102.2	**191.7 ± 123.2**	235.6 ± 104.9	274.8 ± 98.8	195.9 ± 89.0	238.9 ± 96.8
Zn	81.0 ± 28.8	69.9 ± 33.1	75.1 ± 28.8	65.5 ± 7.7	**83.0 ± 29.1**	74.3 ± 28.7
Zr	**498.5 ± 454.2**	398.8 ± 158.2	528.7 ± 221	424.8 ± 31.4	373.8 ± 201.4	508 ± 318.2

[a] Fe concentrations are expressed as (g/kg), [1]detected only once. ND=Not detected
bold = elevated concentrations compared to other stations

3.1.5.1 Elemental associations

Correlation matrices were used to examine the inter-relationship among the investigated metal concentrations. The values of Pearson correlation coefficient (r^2) between metal concentrations are given in Table 3.7. Sulphur, nickel and molybdenum were not involved in the correlation matrix, as they were only detected ≤ 6 times out of 41 samples which were screened (Fig. 3.3). In general, heavy metals, such as Mn, Co, Cu and Zn, were significantly correlated to each other ($p<0.05$). For instance, Co was correlated to Cu and Mn with r^2 of 0.60 and 0.74, respectively. Fe was correlated to Mn and Co with r^2 of 0.76 and 0.68, respectively. Moreover, titanium was negatively correlated to Rb, Sr and Zr with r^2 of -0.51, -0.70 and -0.54, respectively (Table 3.7).

Table 3.7: Pearson correlation matrix showing correlation coefficients values (r) between 16 different metals detected in 38 sediment and soil samples from the Kilombero Valley wetlands ecosystem.

Metal	**K**	**Ca**	**Ti**	**V**	**Cr**	**Mn**	**Fe**	**Co**	**Cu**	**Zn**	**As**	**Rb**	**Sr**	**Zr**	**Pb**	**Th**
K	1.00															
Ca	**0.62****	1.00														
Ti	**-0.61****	-0.40	1.00													
V	0.13	-0.10	0.42	1.00												
Cr	-0.17	0.07	0.40	0.13	1.00											
Mn	-0.02	0.05	0.49*	0.51*	0.36	1,00										
Fe	-0.24	-0.02	**0.70****	**0.61****	0.49*	**0.76****	1.00									
Co	-0.17	0.00	0.47*	0.39	0.32	**0.74****	**0.68****	1.00								
Cu	-0.35	0.04	0.28	0.32	0.20	0.41	0.44	**0.60***	1.00							
Zn	-0.26	-0.02	0.38	**0.62****	0.14	0.23	0.57	0.26	**0.70****	1.00						
As	0.23	-0.01	-0.08	-0.03	-0.17	0.12	-0.14	0.05	-0.26	-0.42	1.00					
Rb	**0.61****	0.19	**-0.51***	0.00	-0.38	-0.08	-0.33	-0.08	-0.24	-0.23	0.55*	1.00				
Sr	**0.66****	0.26	**-0.70****	-0.17	-0.25	-0.24	-0.53	-0.28	-0.41	-0.45*	0.51*	**0.86****	1.00			
Zr	0.49*	0.10	**-0.54***	-0.40	-0.16	-0.38	**-0.64****	-0.45*	-0.54*	**-0.54***	0.19	0.46	**0.71****	1.00		
Pb	-0.18	-0.35	0.08	0.39	-0.40	-0.12	0.07	-0.03	0.09	0.35	-0.12	0.02	-0.10	-0.15	1.00	
Th	-0.51*	-0.38	0.39	0.32	-0.11	-0.03	0.18	0.19	0.35	0.45*	-0.24	-0.27	-0.44	-0.47	**0.59***	1.00

*No. of observations: 38: * Correlation is significant at the 0.05 level, ** Correlation is significant at the 0.01 level. **Bold**=significant correlation*

3.1.5.2 Comparison of the metal screening results from XRFA and ICP-OES

The results of elemental content screening accomplished by portable XRFA for fine dust-fraction (<20 μm) of sediment and soil samples were compared with those obtained by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) using digested samples and certified reference materials. The ICP-OES measurement of the elements listed in the Certified Reference Material (CRM) were comparable with the reference values in the 1:10 diluted samples and percentage recoveries varied according to wavelength of the analyzed metals. Each element was measured at two different wavelengths (nm) i.e. Fe (259.939 nm, 238.204 nm), Mn (259.372 nm, 257.610 nm) Cu (324.752 nm, 327.393 nm), Pb (217.000 nm, 220.353 nm), Cd (214.440 nm, 228.802 nm). Only results from the wavelength and dilution step that showed high percentage recovery of the CRM were selected. The variation of ICP-OES results in different wavelengths and dilutions might be due to spectral interference of the elements and variation in physical characteristics of the samples (sample physical matrix effect). The percentage recoveries for CRM-1 (flooded sediment material) and CRM-2 (loam soils) were also different due to different sample matrices and spectral interference. Therefore, the best recovery wavelengths in 1:10 diluted samples were selected for comparison of the results with XRFA, in accordance with the required limit of detection and the possibility of low interference by other elements present in the digested solution (Table 3.8).

Table 3.8: Percentage recoveries and wavelengths of the selected metals from the reference values of the CRM as detected by ICP-OES

Metal	Wavelength (nm)	Recoveries (%)	
		CRM-1	CRM-2
Fe	238.204	99.88	na
Mn	257.610	99.90	107.59
Cu	327.393	96.54	79.85
Pb	220.353	99.79	114.4
Cd	228.802	91.43	121.68

CRM=certified reference material

Table 3.9 indicates concentrations of each sample under the two analytical methods. The XRFA results in most of the samples were higher than in ICP-OES. Precision and accuracy of the two analytical methods might be affected by spectral interference (peak overlaps) and variation in the physical characteristics of the samples. However, the differences between the concentrations of detected elements by XRFA differ in a mean absolute magnitude value of ± 13.4% from those of ICP-OES as shown in Table 3.10

Table 3.9: Comparison of XRFA and ICP-OES detected concentrations (mg/kg DW) of selected heavy metals that were in agreement with the known concentrations of the CRM.

sample name	Fe (238.204 nm)		Mn (257.610 nm)		Cu (327.393 nm)		Pb (220.353 nm)		Cd (228.802 nm)	
	XRFA	**ICP-OES**	**XRFA**	**ICP-OES**	**XRFA**	**ICP-OES**	**XRFA**	**ICP-OES**	**XRFA**	**ICP-OES**
IP1 [a]	51251	46219.6	3280	3207.0	61	62.6	23	19.6	ND	2.02
IP10 [b]	43075	37857.3	836	307.3	29	38.1	22	48.3	ND	2.16
IP11 [b]	61482	49411.8	1148	910.6	49	51.6	21	27.5	ND	2.08
IP3	78428	50294.2	1285	1044.9	63	52.4	23	41.8	ND	1.88
IP2 [a]	76129	65103.6	1347	1152.1	63	63.7	22	9.7	ND	2.02
KP1 [b]	56723	52468.5	532	419.1	49	54.7	28	7.9	ND	1.98
KP2 [b]	49935	48742.3	576	462.6	41	38.8	29	51.4	ND	1.39
KP3	30323	27822.0	799	690.5	29	46.4	29	22.0	ND	2.27
KP4 [b]	25743	24103.8	789	646.1	32	36.1	26	12.6	ND	1.49
KPL12 [a]	33233	29656.4	490	356.3	58	62.0	23	15.6	ND	1.25
KPL17 [a]	41462	35987.9	711	572.0	80	76.8	20	ND	ND	2.41
KPL19 [b]	52129	45027.7	827	634.7	65	64.1	19	5.2	ND	2.09
KPL4 [a]	19241	12544.9	277	177.5	36	32.6	25	9.0	ND	1.66
KPL5 [a]	29724	29901.9	252	176.8	49	56.2	30	8.2	ND	2.04
KPL6 [a]	25309	25192.0	573	496.8	47	55.4	30	12.4	ND	1.97
KPL7 [a]	29891	24136.0	435	279.8	34	32.6	20	6.9	ND	1.28
KSC1 [a]	28695	24869.0	308	201.5	33	28.8	18	22.7	ND	1.57
KSC15 [a]	18921	16171.9	583	228.2	33	44.1	23	1.0	ND	2.07
KSC2 [a]	54228	46800.0	1051	887.0	47	59.0	19	24.7	ND	2.11
KSC3 [a]	52845	45905.6	805	645.3	37	57.3	20	35.0	ND	2.32
KSC4 [a]	17868	18787.4	178	546.1	8	40.3	8	30.0	ND	1.86
KSC5 [a]	75521	61901.0	796	623.0	52	55.9	20	36.5	ND	2.22
KSC6 [a]	38827	41438.2	698	706.6	40	57.0	21	27.9	ND	2.18
KSC8 [a]	60363	45509.9	777	568.6	46	53.6	22	22.9	ND	2.42
KSC9 [a]	53286	44870.7	658	529.1	64	68.1	22	32.8	ND	2.10
MBS1 [a]	40635	38493.9	704	624.9	40	45.1	23	48.8	ND	1.48
MBS10 [b]	41478	20904.3	541	370.6	78	42.0	32	29.7	ND	1.71
MBS6 [b]	71068	58496.3	941	739.6	57	61.0	25	36.2	ND	2.26
MBS7 [b]	24135	21709.0	495	991.1	36	53.8	22	24.5	ND	2.45
MBS8 [b]	14179	13187.3	274	199.7	26	29.7	22	34.2	ND	1.78
MBS9 [b]	15114	16823.9	259	244.1	27	44.2	20	21.7	ND	1.91
TAC2 [a]	64708	61754.7	944	844.7	49	53.1	26	30.3	ND	1.42
TAC4 [b]	76487	59616.4	1036	802.0	63	62.6	29	53.4	ND	2.29
TAC5 [b]	68560	57423.6	878	726.2	51	67.1	26	19.7	ND	2.52
TAC6 [b]	69801	56893.6	881	753.0	50	57.1	29	41.5	ND	2.14
TAC8 [b]	27444	25482.4	402	340.1	55	61.5	23	14.4	ND	2.09
CRM-1	NA	28365.0	NA	836.1	NA	82.1	NA	138.7	NA	1.82
CRM-2	NA	37511.1	NA	797.3	NA	133.4	NA	95.0	NA	1.75

a=sediment, b=soil, NA=Not available, ND= not detected, Certified Reference Material (CRM-1= Flood sediment material, CRM-2= Loam soils).

Table 3.10: Percentage deviation of XRFA from ICP-OES detected concentrations of selected metals in sediment and soil samples. Negative values means XRFA detected concentrations were lower than those of ICP-OES.

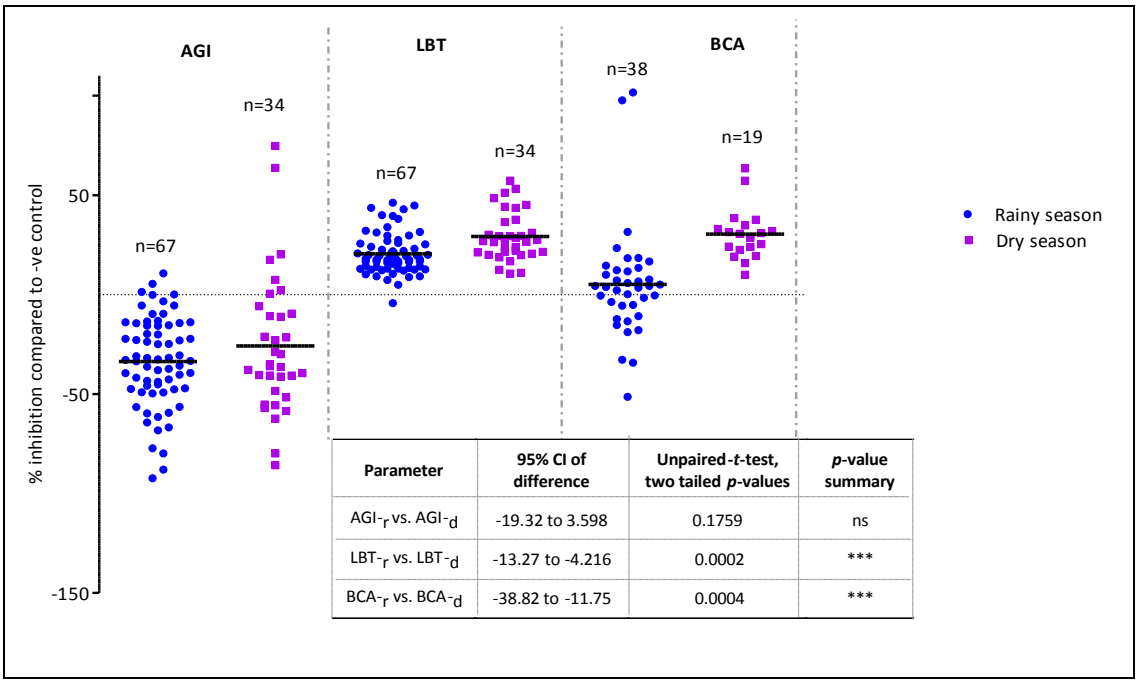
Sample names	Fe (%)	Mn (%)	Cu (%)	Pb (%)
IP1[a]	9.8	2.2	-2.5	14.9
IP10[b]	12.1	63.2	-31.2	*
IP11[b]	19.6	20.7	-5.4	-30.9
IP3	35.9	18.7	16.8	-81.9
IP2[a]	14.5	14.5	-1.2	56.0
KP1[b]	7.5	21.2	-11.7	71.7
KP2[b]	2.4	19.7	5.4	-77.3
KP3	8.2	13.6	-59.9	24.2
KP4[b]	6.4	18.1	-12.9	51.6
KPL12[a]	10.8	27.3	-6.9	32.1
KPL17[a]	13.2	19.5	4.0	na
KPL19[b]	13.6	23.3	1.4	72.6
KPL4[a]	34.8	35.9	9.3	64.2
KPL5[a]	-0.6	29.8	-14.6	72.7
KPL6[a]	0.5	13.3	-17.9	58.6
KPL7[a]	19.3	35.7	4.1	65.6
KSC1[a]	13.3	34.6	12.7	-25.9
KSC15[a]	14.5	60.9	-33.6	95.8
KSC2[a]	13.7	15.6	-25.5	-29.7
KSC3[a]	13.1	19.8	-54.8	-74.9
KSC4[a]	-5.1	*	*	*
KSC5[a]	18.0	21.7	-7.5	-82.3
KSC6[a]	-6.7	-1.2	-42.6	-32.9
KSC8[a]	24.6	26.8	-16.5	-4.1
KSC9[a]	15.8	19.6	-6.4	-49.1
MBS1[a]	5.3	11.2	-12.9	*
MBS10[b]	49.6	31.5	46.2	7.1
MBS6[b]	17.7	21.4	-7.1	-44.8
MBS7[b]	10.1	*	-49.6	-11.2
MBS8[b]	7.0	27.1	-14.3	-55.5
MBS9[b]	-11.3	5.7	-63.7	-8.7
TAC2[a]	4.6	10.5	-8.3	-16.6
TAC4[b]	22.1	22.6	0.7	-84.0
TAC5[b]	16.2	17.3	-31.5	24.0
TAC6[b]	18.5	14.5	-14.1	-42.9
TAC8[b]	7.1	15.4	-11.8	37.3

a=sediment, b=soil, na = not available because it was not detected by one method,
** ICP-OES concentration is more than two times higher than XRFA.*

3.2 Eco-toxicity of environmental samples using standardized bioassays

3.2.1 Rainy versus dry season (sediment, soil and water samples)

There were no significant differences in the inhibition or stimulation of *P. subcapitata* auto-fluorescence exhibited by dry and rainy season samples (p=1.759). 34% and 26% mean stimulation of *P. subcapitata* bioassay were shown by dry and rainy season samples (sediments, soil and water), respectively (Fig. 3.4). Dry season samples (sediment, soil, and water) exhibited a significantly higher mean inhibition (29%) than rainy season samples (18%) for *V. fischeri* (p=0.0002). Moreover, dry season samples showed significantly higher mean inhibition of 31%, for the *A. globiformis* bioassay, as opposed to 5% caused by rainy season samples (p=0.0004). Details of the impact of sediment, water and soil samples to each test organisms are discussed separately in Section 3.2.2. In general, dry season samples showed higher mean inhibition of bioassays endpoints than the rainy season samples (sediment, soil, water).



Parameter	95% CI of difference	Unpaired-*t*-test, two tailed *p*-values	*p*-value summary
AGI_r vs. AGI_d	-19.32 to 3.598	0.1759	ns
LBT_r vs. LBT_d	-13.27 to -4.216	0.0002	***
BCA_r vs. BCA_d	-38.82 to -11.75	0.0004	***

Figure 3.4: Scatter plot of inhibition of all biotests data (sediment, soil and water) of test organisms exposed to Kilombero Ramsar Site samples. The horizontal line within the points indicates the mean value response, and the inserted table indicates statistical analysis results. AGI= Algae growth inhibition test, LBT=Luminescence bacteria test, BCA= Bacteria contact assays, r= rainy season, d=dry season, n= number of samples

3.2.2 Eco-toxicity of sediments, soil and water matrices

3.2.2.1 Sediment samples

For all three bioassays, sediment samples collected during the dry season (n=19) exhibited higher inhibitions of measured bioassays endpoints than sediment samples collected during the rainy season (n=39). As shown in Fig. 3.5, when *P. subcapitata* was exposed to sediment elutriates collected during rainy season (n=39), 3 of the samples resulted in inhibition with a maximum of 5.4% while 36 of the samples caused mean and maximum stimulation of 30% and 92%, respectively. Two rainy season sediment samples, each from TAC and KSC, showed highest stimulation of *P. subcapitata* bioassay by 90.91% and 92.32%, respectively. Additionally, 6 out of 19 dry season sediment samples showed inhibition of *P. subcapitata.* One dry season sediment sample from KSC, out of the six samples, which showed inhibition, had the highest inhibition of *P. subcapitata* by 74%, while 13 samples out of 19 dry season sediment samples, resulted in stimulation varying between 3.37% and 85%. Therefore, mean stimulations of *P. subcapitata* were 30% and 19% by rainy and dry seasons sediment elutriates, respectively. Additionally, when *V. fischeri* was exposed to rainy season sediment elutriates (n=39), inhibition between 7% and 31% was observed, while dry season sediment elutriates showed an inhibition between 10% and 53% (Fig. 3.5). Neither *P. subcapitata* nor *V. fischeri* bioassays showed any significant differences in the mean inhibitions between rainy and dry seasons ($p>0.05$, Dunn´s multiple comparison test). Besides, there were significant differences between the inhibition shown by dry and rainy season sediments for *A. globiformis* bioassay ($p<0.001$). 14 sediments out of 39 rainy season sediment samples showed stimulation with a maximum of 34%, while no dry season sediment samples (n=19) showed stimulation in the *A. globiformis* bioassay.

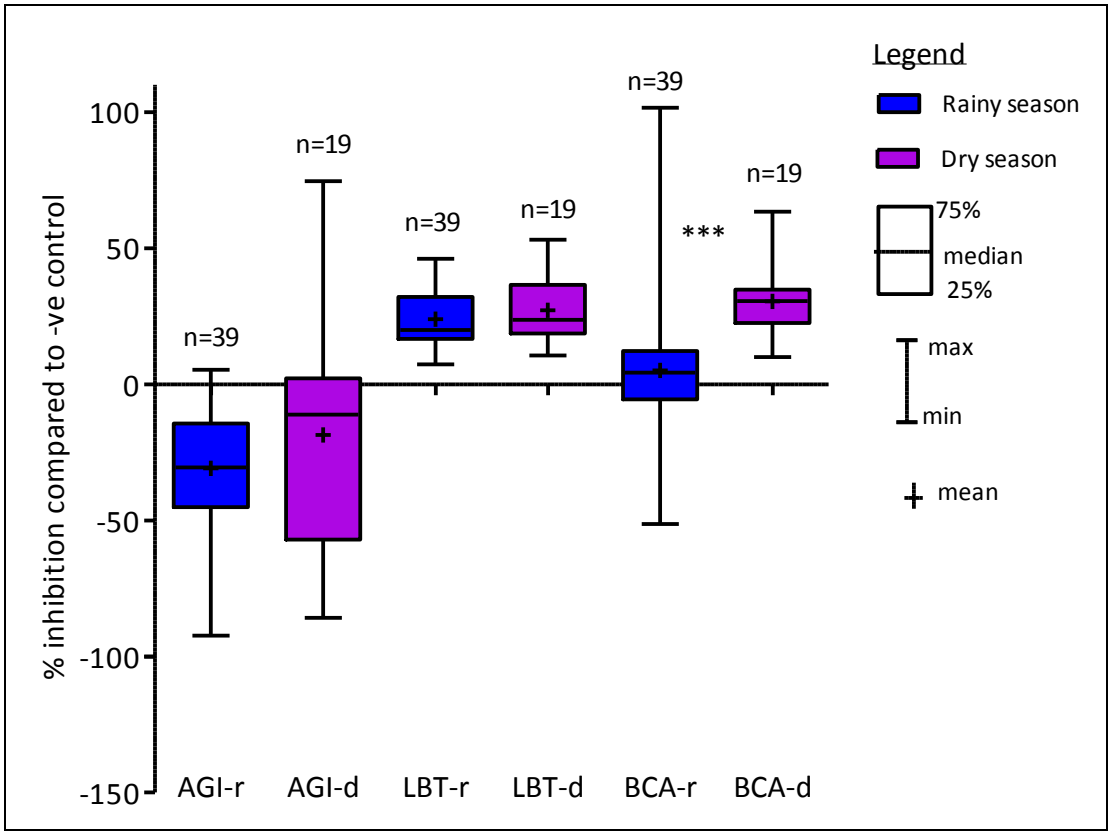


*Figure 3.5: Box-whisker plots indicating the mean, minimum and maximum inhibition of measured responses of test organisms exposed to sediment samples. AGI= Algae growth inhibition test, LBT=Luminescence bacteria test, BCA= Bacteria contact assays, r= rainy season, d=dry season, ***:* p*<0.001, 1-way ANOVA, with post-test Dunn´s multiple comparison test*

3.2.2.2 Water samples

40 out of 41 and 25 out of 27, rainy and dry season water samples respectively, resulted in stimulation of *P. subcapitata*. (Table 3.11). The maximum inhibition of *P. subcapitata* by rainy and dry season samples was 10.7% and 63.8%, respectively (Fig. 3.6). Only 1 out of 41 rainy season water samples showed 4.3% stimulation while the rest of rainy and all dry season water samples (n=67) showed inhibition of *V. fischeri* (Fig. 3.6). However, neither *P. subcapitata* nor *V. fischeri* bioassays had any significance differences in the mean inhibitions between rainy and dry seasons ($p>0.05$, Dunn´s multiple comparison test).

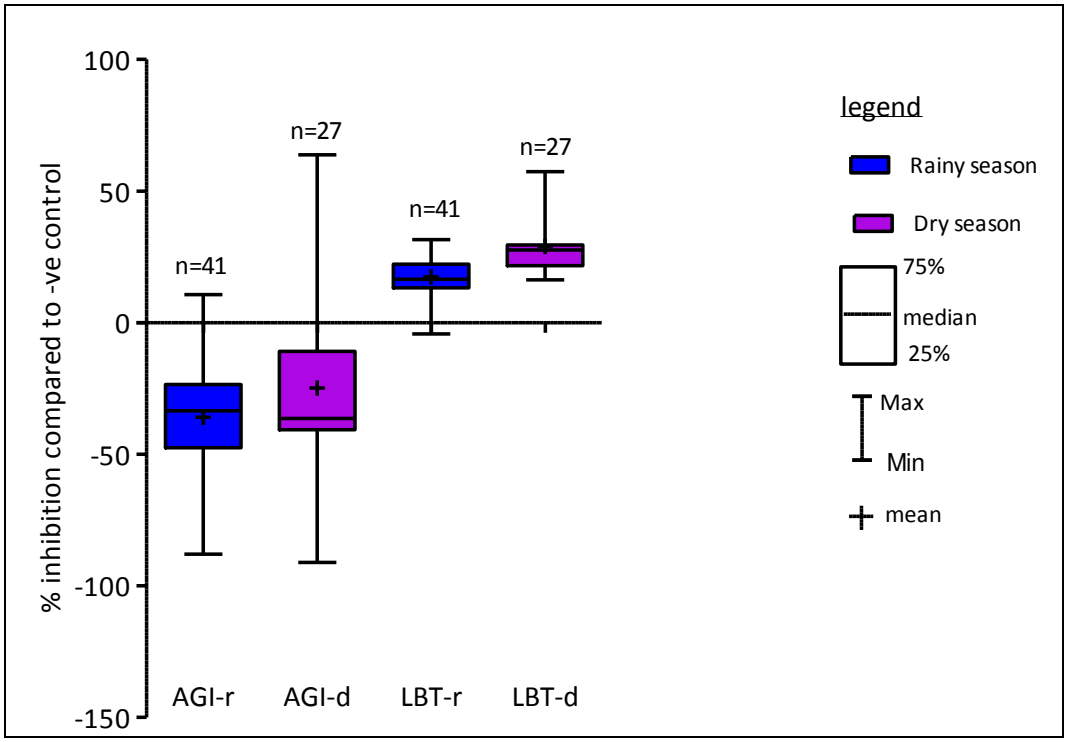


Figure 3.6: Box-whisker plots indicating the mean, minimum and maximum percentage inhibition of measured responses of test organisms exposed to water samples. AGI= Algae growth inhibition test, LBT=Luminescence bacteria test, r= rainy season, d=dry season

3.2.2.3 Soil samples

There was no inhibition of *P. subcapitata* by dry season soil samples (n=17). The dry season soil samples (n=17) exhibited 69% mean stimulation of *P. subcapitata*, but 34% and 25% mean inhibitions of *V. fischeri* and *A. globiformis*, respectively (Table 3.11, Fig. 3.7). Only 1 out of 17 dry season soil samples resulted in a stimulation of 5.7% of *A. globiformis*.

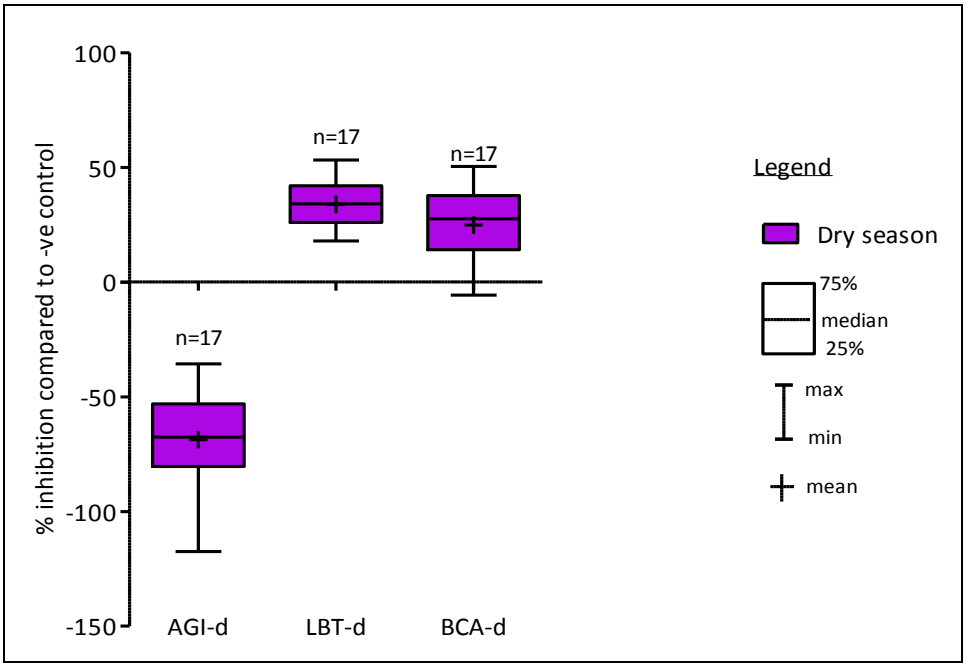


Figure 3.7: Box-whisker plots indicating the mean, minimum and maximum values of percentage inhibition of bioassay measured responses of test organisms exposed to soil samples. AGI= Algae growth inhibition Test, LBT=Luminescence bacteria test, BCA= Bacteria contact assays, d=dry season.

In summary, different sample matrices resulted in different biotest responses. One-way ANOVA revealed a statistically significant difference in the endpoint responses of the three bioassays (p<0.001) and between sediment and water samples (p<0.005). There is a noticeable variation between the tests, among the various samples and even amongst the two sampling seasons.

Table 3.11: Summary of the measured endpoints responses of different bioassays to sediment, water and soil samples collected during rainy and dry season.

Biotests	Test organism	Measured endpoint response	Samples-matrix	Sampling season	Mean % inhibition	Remark
AGI	*P. subcapitata*	Growth rate	Water	Rainy	-35.9	Stimulation
				Dry	-24.9	Stimulation
			Sediment elutriate	Rainy	-30.91	Stimulation
				Dry	-18.6	Stimulation
			Soil elutriate	Dry	-68.7	Stimulation
LBT	*V. fischeri*	Metabolic activity	Water	Rainy	17.4	Inhibition
				Dry	28.9	Inhibition
			Sediment elutriate	Rainy	17.4	Inhibition
				Dry	27.2	Inhibition
			Soil elutriate	Dry	33.9	Inhibition
BCA	*A. globiformis*	Dehydrogenase activity	Sediment slurries	Rainy	-5.16	slight stimulation
				Dry	30. 5	Inhibition
			Soil slurries	Dry	24.9	Inhibition

3.3 Classification of results to integrate toxicity responses

3.3.1 Toxicity categorization and fuzzy classes

Table 3.12 presents estimates of the fuzzy overlap for each biotest result. The fuzzy overlaps were derived from the average standard deviation of the positive and negative controls, and from the mean standard deviation of the test replicates. Average standard deviations among the replicates of positive and negative controls were used to derive fuzzy overlaps for each biotests.

Table 3.12: Estimation of the size of overlaps between the toxicity categories based on the standard deviation of inhibition values of replicates of positive and negative controls

Biotest	Average SD of replicates	Average SD of positive control	Average SD of negative control	Fuzzy overlaps
AGI (elutriate)	<5%	<10%	<5%	10%
LBT (elutriate)	<3%	<10%	<3%	10%
BCA (slurries)	10%	<25%	*Ca*.5%	25%

SD of negative controls and of replicates based on the validity criteria of each bioassay.

The contribution of the three bioassays, for each sample, to the three categories is summarized in Table 3.13. The membership functions, derived from the measured end-points responses categories (i.e. no or low toxic responses, moderate toxic responses and high toxic responses), indicate to what extent the bioassay data belong to a particular fuzzy set (Figs. 3.8, 3.9 and 3.10). The membership functions of bioassays, defined by any value between 0 and 1, were used to obtain three fuzzy quality classes, namely; Class 1: Little or no potential risk, Class 2: Critical risk, Class 3: Elevated critical risk.

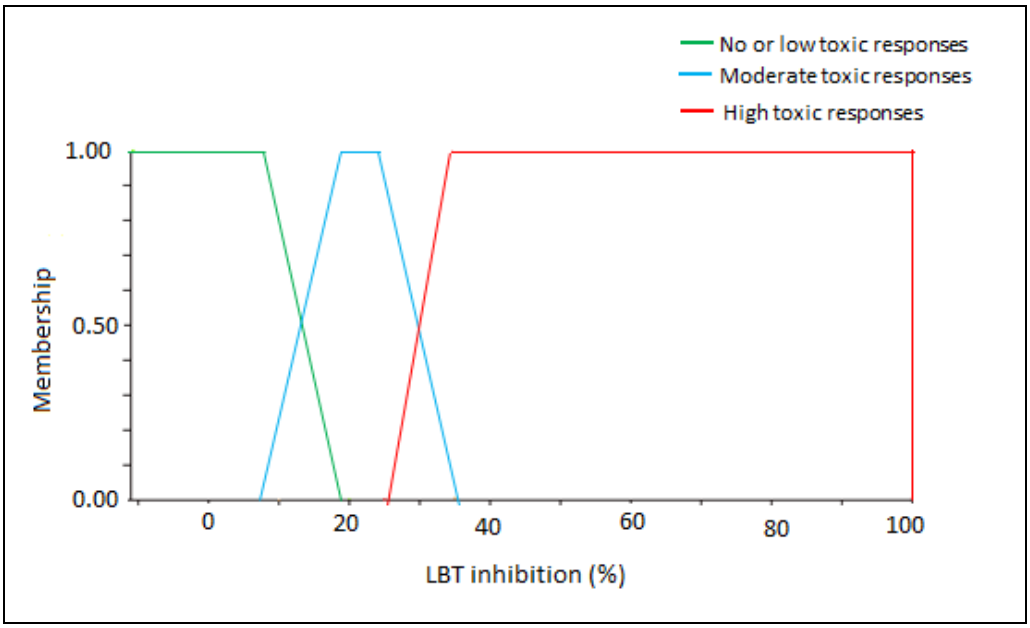


Figure 3.8: Membership functions of the fuzzy sets for luminescent bacteria test (LBT). Fuzzy overlaps are derived from standard deviation of inhibition values of replicates of positive and negative controls

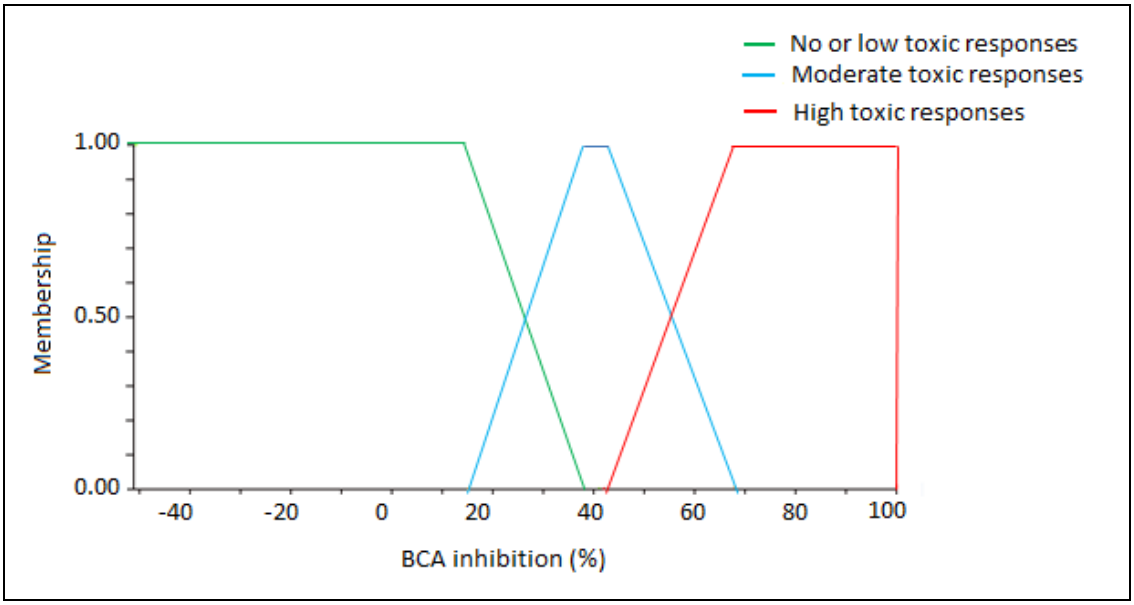


Figure 3.9: Membership functions of the fuzzy sets for Bacteria Contact Assay (BCA). Fuzzy overlaps are derived from standard deviation of inhibition values of replicates of positive and negative controls

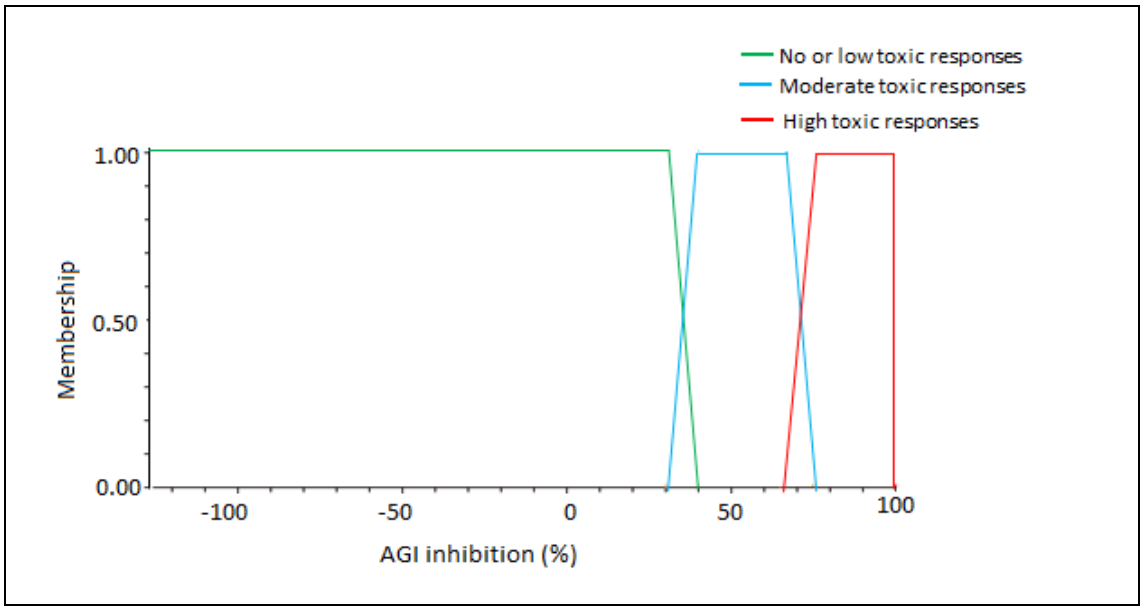


Figure 3.10: Membership functions of the fuzzy sets for the Algae Growth Inhibition test (AGI). Fuzzy overlaps are derived from standard deviation of inhibition values of replicates of positive and negative controls

Table 3.13: Overview of weight contribution of each bioassay to the toxicity classes based on the fuzzy rules assigned to 75 total samples (58-sediments and 17-soils) collected during dry and rainy seasons.

sample Name	Bioassays % inhibition compared to negative controls			weight contribution to the toxicity response or category				
	AGI	LBT	BCA	Class 1.Little or no potential risk	Class 2: Critical risk	Class 3.Elevated Critical risk	Assigned final Class	station
CH1*	-41.76	18.33	-15.30	0.83	0.00	0.00	1	Chita NS
CH2*	-64.24	21.84	-18.89	1.00	0.00	0.00	1	Chita NS
CH3*	-37.34	10.18	-12.31	0.98	0.00	0.00	1	Chita NS
CH4*	-43.40	16.70	-34.19	0.67	0.320	0.00	1	Chita NS
IP1	20.19	22.10	30.90	0.39	0.61	0.00	2	IP
IP2	-10.84	26.81	57.30	0.37	0.63	0.18	2	IP
IP3	7.39	51.29	24.20	0.65	0.35	0.00	1	IP
IP4*	-45.84	19.52	-3.68	0.95	0.05	0.00	1	IP
IP5*	-23.70	17.72	-1.61	0.77	0.28	0.00	1	IP
IP6*	-0.07	27.45	-0.53	0.76	0.230	0.00	1	IP
IP7*	-40.23	31.26	-5.21	0.63	0.00	0.00	1	IP
IP8*	1.39	25.93	16.61	0.91	0.06	0.00	1	IP
IP9	-54.22	30.10	37.8	0.12	0.51	0.00	2	IP
IP10	-48.34	17.87	28.4	0.48	0.52	0.00	2	IP
IP11	-35.69	26.23	50.4	0.10	0.88	0.10	2	IP
KP1	-51.99	20.95	28.6	0.48	0.52	0.00	2	KP
KP2	-86.04	45.78	10.9	1.00	0.00	0.00	1	KP
KP3	-69.92	25.88	16.9	0.91	0.07	0.00	1	KP
KP4	-74.81	43.32	48.5	0.00	0.98	0.02	2	KP
KPL1	-21.12	43.64	38.50	0.10	0.90	0.00	2	KPL
KPL2	-9.55	30.17	34.80	0.44	0.52	0.00	2	KPL
KPL3	-29.93	10.97	19.40	0.83	0.10	0.00	1	KPL
KPL4	2.21	16.73	28.60	0.48	0.52	0.00	2	KPL
KPL5	-62.25	20.59	31.40	0.37	0.63	0.00	2	KPL
KPL6	-79.83	12.51	31.90	0.65	0.25	0.00	1	KPL
KPL7*	-56.43	39.57	3.80	1.00	0.00	0.00	1	KPL
KPL8*	-59.46	44.77	-51.34	1.00	0.00	0.00	1	KPL
KPL9*	-37.97	24.12	4.39	1.00	0.00	0.00	1	KPL
KPL10*	-13.51	29.67	101.66	0.18	0.81	0.00	2	KPL
KPL11*	15.37	14.41	97.62	0.44	0.56	0.00	2	KPL
KPL12*	-9.78	42.46	5.24	1.00	0.00	0.00	1	KPL
KPL13*	-30.56	16.76	-13.45	0.68	0.32	0.00	1	KPL
KPL14*	-22.21	9.25	7.58	1.00	0.00	0.00	1	KPL
KPL15*	-20.05	12.57	4.33	0.74	0.230	0.00	1	KPL
KPL16*	-23.01	13.08	-5.53	0.69	0.29	0.00	1	KPL
KPL17*	-5.46	23.12	-17.90	1.00	0.00	0.00	1	KPL
KPL18*	-5.44	17.40	-32.70	0.74	0.26	0.00	1	KPL

Table 3.13 continued

KPL19	-65.87	40.20	13.3	1.00	0.00	0.00	1	KPL
KSC1	-85.82	53.19	24.00	0.65	0.35	0.00	1	KSC
KSC2	-57.08	23.65	33.00	0.31	0.69	0.00	2	KSC
KSC3	-58.57	36.52	10.00	1.00	0.00	0.00	1	KSC
KSC4	-21.51	19.86	37.70	0.13	0.87	0.00	2	KSC
KSC5	-11.13	21.25	30.60	0.40	0.60	0.00	2	KSC
KSC6	-5.89	10.64	25.40	0.60	0.26	0.00	1	KSC
KSC7	-22.79	26.33	15.80	0.87	0.12	0.00	1	KSC
KSC8	0.43	18.72	22.50	0.71	0.29	0.00	1	KSC
KSC9	74.71	26.47	63.40	0.00	0.31	0.62	3	KSC
KSC10*	-39.48	15.87	6.66	0.69	0.30	0.00	1	KSC
KSC11*	-30.94	7.35	31.52	0.64	0.33	0.00	1	KSC
KSC12*	5.40	19.97	11.71	1.00	0.00	0.00	1	KSC
KSC13*	-15.61	19.95	0.15	1.00	0.00	0.00	1	KSC
KSC14*	0.12	18.99	-10.86	0.90	0.00	0.00	1	KSC
KSC15*	-22.85	17.30	13.47	0.73	0.00	0.00	1	KSC
KSC16*	-66.72	17.99	18.29	0.80	0.13	0.00	1	KSC
KSC17*	-92.34	8.78	18.39	0.87	0.00	0.00	1	KSC
MBS1	17.52	45.08	19.10	0.84	0.16	0.00	1	MBS
MBS2*	-15.66	46.16	7.14	1.00	0.00	0.00	1	MBS
MBS3*	-59.73	42.93	-0.38	1.00	0.00	0.00	1	MBS
MBS4*	-14.39	19.20	23.38	0.68	0.32	0.00	1	MBS
MBS5*	-14.90	38.08	14.54	1.00	0.00	0.00	1	MBS
MBS6	-67.55	27.69	37.7	0.13	0.73	0.00	2	MBS
MBS7	-64.02	26.55	27.6	0.52	0.48	0.00	1	MBS
MBS8	-62.30	53.24	-5.7	1.00	0.00	0.00	1	MBS
MBS9	-117.43	44.17	3.2	1.00	0.00	0.00	1	MBS
MBS10	-69.32	39.79	14.8	1.00	0.00	0.00	1	MBS
TAC1*	-49.16	33.91	12.29	0.89	0.10	0.00	1	TAC
TAC2*	-68.20	32.06	3.46	0.71	0.28	0.00	1	TAC
TAC3*	-24.93	39.91	10.03	1.00	0.00	0.00	1	TAC
TAC4*	-32.06	43.65	2.14	1.00	0.00	0.00	1	TAC
TAC5	-86.39	34.05	22.8	0.70	0.30	0.00	1	TAC
TAC6	-50.62	40.60	29.7	0.43	0.57	0.00	2	TAC
TAC7	-90.91	38.17	41.9	0.00	1.00	0.00	2	TAC
TAC8	-73.13	21.35	16.7	0.93	0.07	0.00	1	TAC
TK1*	-33.30	12.72	5.87	0.73	0.27	0.00	1	Teak Co.
TK2*	-45.14	22.02	4.99	1.00	0.00	0.00	1	Teak Co.

Grey shaded sample names=soil samples, * Rainy season sediment samples

In general, three variable attributes (BCA, LBT, and AGI), three toxicity responses (low, moderate and high), and 27 fuzzy based rules (Appendix 5), produced different distribution of fuzzy rule based (FRB) toxic classes among 75 samples (sediment and soils) from Kilombero valley Ramsar Site.

Table 3.14: Results of FRB classification of bioassays results of 75 samples (sediments and soils) for both rainy and dry seasons, classified according to resulted toxic responses.

Assigned FRB class	**Rainy** season	**Dry** season	**Total** samples	% class contribution (n= 75)
Class 1: Little or no potential risk	37	18	46	73%
Class 2: Critical risk	2	17	11	25%
Class 3: Elevated critical risk	0	1	1	1%
Total samples	39	36	75	100%

Table 3.14 indicates that of all 75 samples (sediment and soils) collected in both rainy and dry season, 73% showed little or no toxic responses to the test organisms, thus assigned into class 1 (little or no potential risk), while 25% of samples, contributed to Class 2 (critical risks), and only 1% resulted in class 3 (elevated critical risk). An overview of the distribution of toxic classes is shown in Fig.3.11.

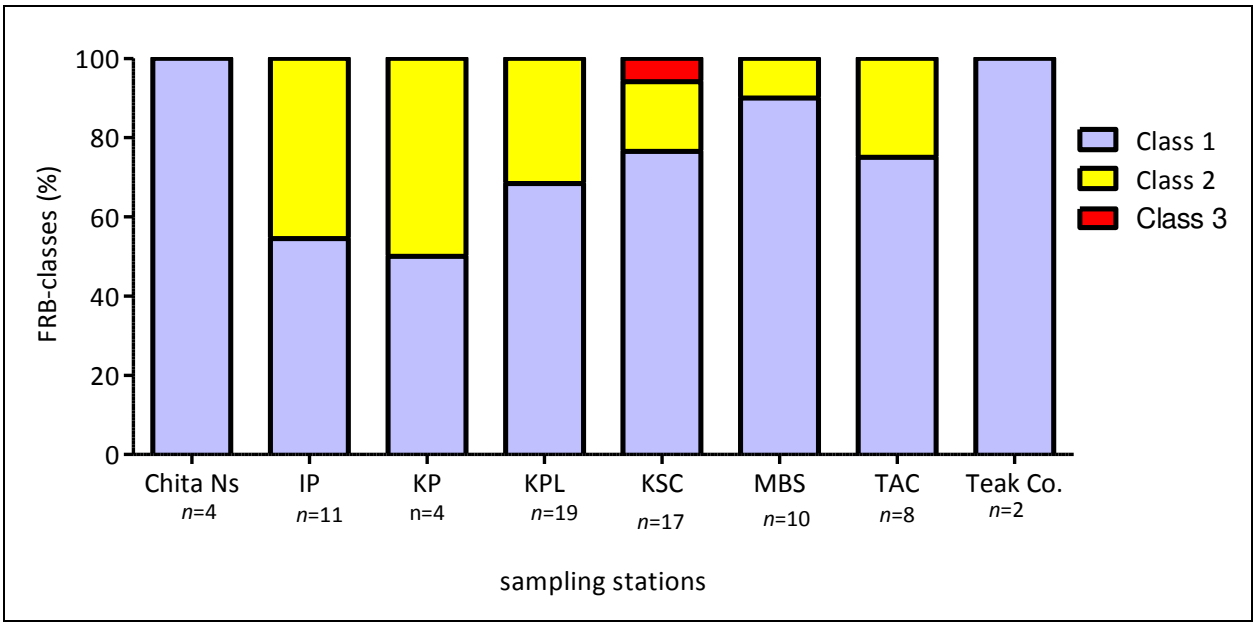


Figure 3.11: Overview of distribution of FRB classes of bioassays results of 75 samples (sediments and soils) for both rainy and dry season. Class 1= little or no potential risk, Class 2= Critical risk, Class 3=Elevated critical risk.

Figure 3.11 indicates that 100% of sediment samples from two stations, Chita Ns and Teak Co, resulted in class 1, "little or no potential risk" while the rest of six sampling stations showed varying proportions of both class 1 and class 2 "critical risk". 6% of KSC sediment samples (n=17) resulted in class 3, "elevated critical risk" whereby high toxic or inhibition responses to the three bioassays were observed. A sample could show low or moderate toxic response in one test, and on the

contrary, showed toxic response to another bioassay. Low toxic responses from LBT, BCA and AGI, due to algae stimulatory values skewed the classification system into class 1, while moderate toxic responses from BCA and LBT, contributed to class 2, and high toxic responses to the three bioassays contributed to class 3.

3.3.2 Relationship between fuzzy classes, bioassay results and chemical data

Table 3.15 summarizes bioassays results and fuzzy rule base (FRB) classes, in relation to detected pesticides and C/N ratio of different sampling stations, season and sample matrices

Table 3.15: Relationship between inhibition of bioassay response to pesticides and C/N ratios of sediment and soil samples, (where pesticides were detected), for a particular season and sampling station

Sample	Bioassays (% inhibition of endpoint response)			Concentration of detected pesticides (µg/g for sediments or soils, and µg/l for water samples)	C/N ratio	Assigned FRB class	sample matrices
	AGI	LBT	BCA				
IP3	7.39	**51.29**	**24.2**	glyphosate (0.092), AMPA (0.08)	14.08	2	Sed[1]
IP2	-10.84	**26.81**	**57.3**	glyphosate (0.045), AMPA (0.043)	13.12	2	Sed[1]
IP9	-54.22	**30.1**	**37.8**	glyphosate (0.05)	15.1	1	soil[1]
IP11	-35.69	**26.23**	**50.4**	glyphosate (0.55), AMPA (0.49)	13.9	2	soil[1]
KP4	-74.81	**43.32**	**48.5**	glyphosate (0.01), AMPA (0.01), diuron (0.952)	17.9	2	soil[1]
KPL11	15.37	14.41	**97.52**	chlorpyrifos (1.83)	na	2	Sed[2]
KPL8	-59.46	**44.77**	**-51.34**	chlorpyrifos (0.12)	17.1	2	Sed[1]
KPL9	-37.97	**24.12**	4.39	AMPA (0.24)	na	1	Sed[2]
KPL19	-65.87	**40.2**	13.2	glyphosate (0.09)	13.2	1	soil[1]
KSC12	5.4	**19.97**	11.71	2-hydroxy-atrazine (0.73)	11.2	1	Sed[2]
KSC2	-57.08	**23.65**	**33**	diuron (1.1), desmethyldiuron (0.096)	9.44	1	Sed[1]
KSC8	0.43	**18.72**	**22.5**	diuron (0.165)	8.95	1	Sed[1]
KSC4	-21.51	**19.86**	**37.7**	diuron(0.033)	10.26	1	Sed[1]
KSC6	-5.89	10.64	**25.4**	diuron 0.063	11.91	1	Sed[1]
KSC5	-11.13	**21.25**	**30.6**	diuron (0.026), desmethyldiuron (0.05)	11.04	1	Sed[1]
KSC15	-22.85	**17.3**	13.47	diuron (0.034)	11.1	1	Sed[2]
KSC9	**74.71**	**26.47**	**63.4**	glyphosate (0.19), atrazine (0.46)	22.1	3	Sed[1]
KSC-w1	10.68	13.01	na	diuron (0.2),desmethyldiuron (0.053), ametryn (0.08), atrazine (0.11), hexazinone (0.097), metribuzin (0.182), propoxur (0.065)	13.5	nc	water[1]
KSC-w2	-3.37	**29.72**	na	diuron (1.7), desmethyldiuron (0.11), 2-hydroxyatrazine (0.11), ametryn (0.097), atrazine (0.52), metribuzin (0.12), monuron (3.9), propoxur (0.083)	16.8	nc	water[2]
KSC-w3	-47.66	12.08	na	diuron (0.053), 2-hydroxyatrazine (0.095), ametryn (0.12), monuron	na	nc	water[2]

				(1.28), propoxur (0.063)			
KSC-w4	-19.76	14.17		atrazine (0.25)	na	nc	water[2]
MBS2	-15.66	**46.16**	7.14	2-hydroxyatrazine (0.56)	17.9	2	Sed[2]
MBS10	-69.32	**39.79**	14.8	glyphosate (0.035)	18.9	1	soil[1]
TAC6	-90.91	**38.17**	**41.9**	AMPA (0.052)	15.1	2	soil[1]

[1] dry season, [2] rainy season, nc = not classified, na = not available, sed = sediment, **bolded** = Moderate or high toxic responses according to FRB toxicity categories, **-ve** values = stimulation of the bioassays response.

There were moderate (15-30%) to high (>30%) inhibition responses for *V. fischeri* by sample matrices in which pesticides, such as chlorpyrifos, 2-hdroxyatrazine, glyphosate and AMPA, were detected (Table 3.15). Moderate (17-60%) to high (>60%) inhibition of *A. globiformis* bioassay was observed in samples in which glyphosate and AMPA (n=6), chlorpyrifos (n=1) and mixture of pesticides such as glyphosate, diuron, desmethydiuron, and atrazine (n=4) were detected. More than 30% stimulation of *P. subcapitata* was shown by sample matrices (n=9), in which glyphosate, AMPA or diuron were detected, while an inhibition of 74.71% was exhibited by one sample (KSC9) in which, a mixture of glyphosate and atrazine were detected. This sample, (KSC9), has a widest measured C/N ratio of 22.1. However, water samples (n=3), in which mixture of pesticides were detected showed little or no toxic response in *P. subcapitata* bioassay. In general, among the samples where pesticides were detected, sediments and soils exhibited a higher toxicity response in one or two of the bioassays than water samples (Table 3.15).

3.4 Evaluation of observed algae growth stimulation in 72-hours AGI

As summarized formerly in Table 3.11 and 3.15, of all rainy and dry season sediments, soil and water samples (n=143), collected from 8 sampling stations: 131 samples (sediment, soil and water) showed stimulation, while 12 samples (sediment, soil and water) showed inhibition of *P. subcapitata* in the chronic, 72-hours exposure. In order to evaluate the potential cause of stimulation in *P. subcapitata* bioassay, the following hypotheses were formulated and tested.

Hypothesis 1: Stimulation of measured algae auto-fluorescence might be due to blockage of continuous electron flow in PS II resulting from the presence of chemicals or pesticides, such as diuron, which interfere with the PS II electron flow system. Fig. 3.12 shows the mode of action of PS II inhibitors. Inhibition of PS II leads to high fluorescence detection (stimulation) due to electrons that fall back when transport system is interfered. Therefore, the hypothesis formulated was, "If *P. subcapitata* is exposed to PS-II inhibitors, then delayed fluorescence (DF) and prompt florescence (PF) measurement will differentiate the pattern of response between the presence of PS II

inhibitors and chemicals with different mode of action". Both chronic and acute tests were carried out using four different chemicals, two with different mode of action and two photosystem II inhibitors. Delayed and prompt fluoresce were measured under acute tests (30 minutes) while only prompt fluorescence was measured under chronic test (72 hours). Different dilution steps (concentrations) of test chemicals were used. Additionally, inhibition of algae auto fluorescence intensity in the chronic tests, were compared with cell density, and fluorescence per cell was computed for each dilution step. Results of the methods used to test this hypothesis are presented in Sections 3.4.1 to 3.4.3.

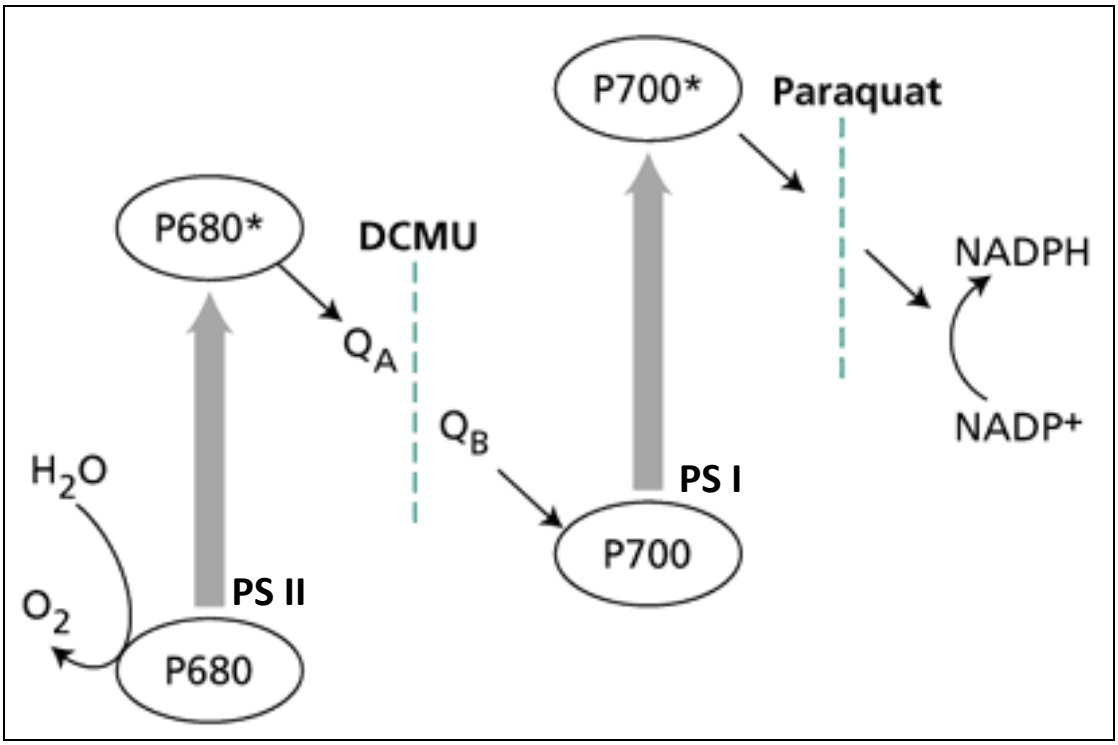


Figure 3.12: Mode of action of phenyl-urea herbicides such as diuron (DCMU), which blocks electron flow at the Quinone acceptors of photosystem II, by competing for the binding site of plastoquinone that is normally occupied by QB (Vogelmann, 2002).

Hypothesis 2: "If elevated nutrients in the aquatic ecosystems enhance algae growth, then excess organic nutrients in the sample elutriates will cause stimulation of *P. subcapitata,* and thus outweigh any toxic effect". Potential nutrients such as nitrogen, phosphate, iron, carbon, vitamins and other micronutrients, were tested. Exponentially growing algae were exposed to a modified DIN-medium, which contained elevated one type of essential nutrient while maintaining the rest of essential nutrients for algae to grow as recommended in the DIN-medium. Serial dilutions produced different concentrations of nutrients. Table 3.22 summarizes the sources of nutrients, positive and negative controls while results are presented in Section 3.4.3.3.

3.4.1 Acute Algae Test: prompt and delayed fluorescence emissions

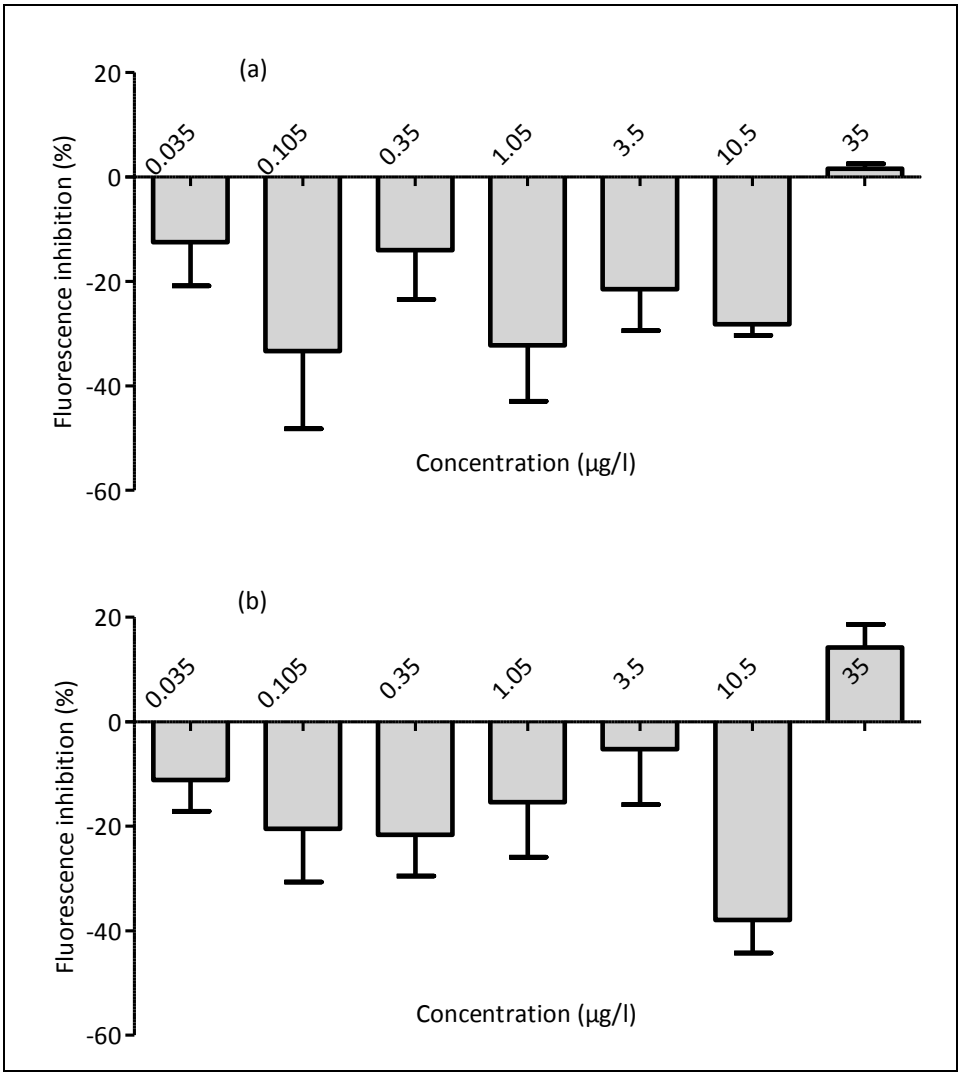


Figure 3.13: (a) Prompt fluorescence and (b) Delayed fluorescence inhibition response after 30 minutes exposure of exponentially growing algae, P. subcapitata to different concentrations of 3,5-DCP. Standard deviation: n=4 repeated experiments

Figure 3.13 shows that the highest concentration, 35 µg/L of 3,5-DCP, resulted in a maximum inhibition of algae auto fluorescence by 1.5% and 14.20% in PF and DF, respectively. Concentrations of 3,5-DCP lower than 35 µg/L resulted in mean stimulation of 20.02% and 13.94% in prompt and delayed fluorescence, respectively. However, there were no significance differences in the observed stimulation between prompt and delayed fluorescence (*p=0.2040 two-tailed, non-parametric, t-test*).

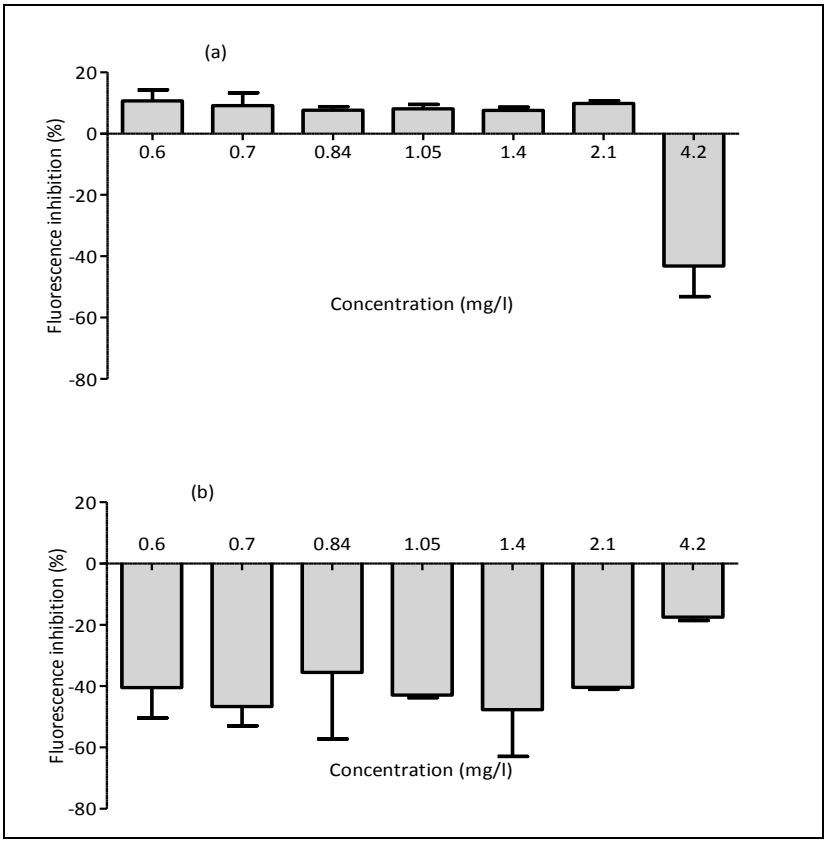


Figure 3.14: (a) Prompt fluorescence and (b) Delayed fluorescence inhibition response after 30 minutes exposure of exponentially growing algae, P. subcapitata to different concentrations of glyphosate-Round-Up. Standard deviation: n=4 repeated experiments

Fig. 3.14 indicates that with an exception of 4.2 mg/L, that resulted in stimulation, all other concentrations of glyphosate (≤2.1 mg/L), showed inhibition of prompt fluorescence emission. All concentrations of glyphosate showed stimulation of delayed fluorescence. There were significant differences in the inhibition of prompt and delayed fluorescence, shown by the same concentration of glyphosate (*p=0.0110, two-tailed, non-parametric, t-test*).

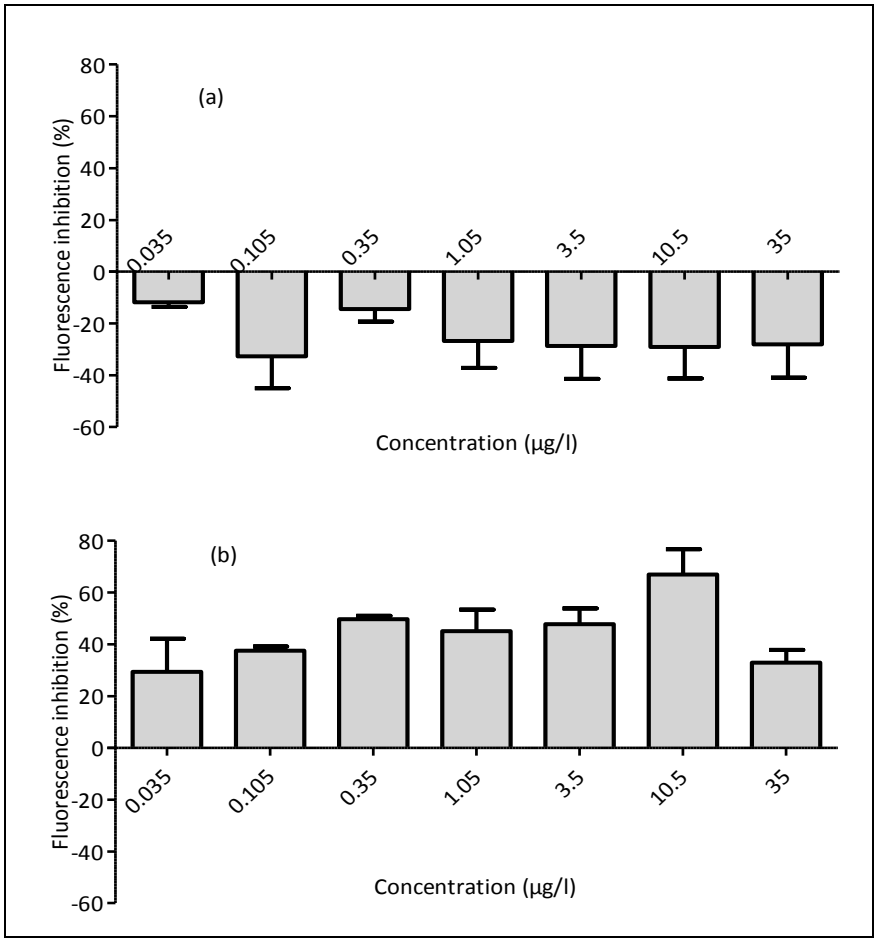


Figure 3.15: (a) Prompt fluorescence and (b) Delayed fluorescence inhibition response after 30 minutes exposure of exponentially growing algae, P. subcapitata to different concentrations of diuron. Standard deviation: n=4 repeated experiments

Diuron, the same as isoproturon, at all concentrations resulted in a statistically significant stimulation of prompt light emission and inhibition of delayed light emission *($p<0.0001$, two tailed, paired t-test,* Fig. 3.15 and 3.16).

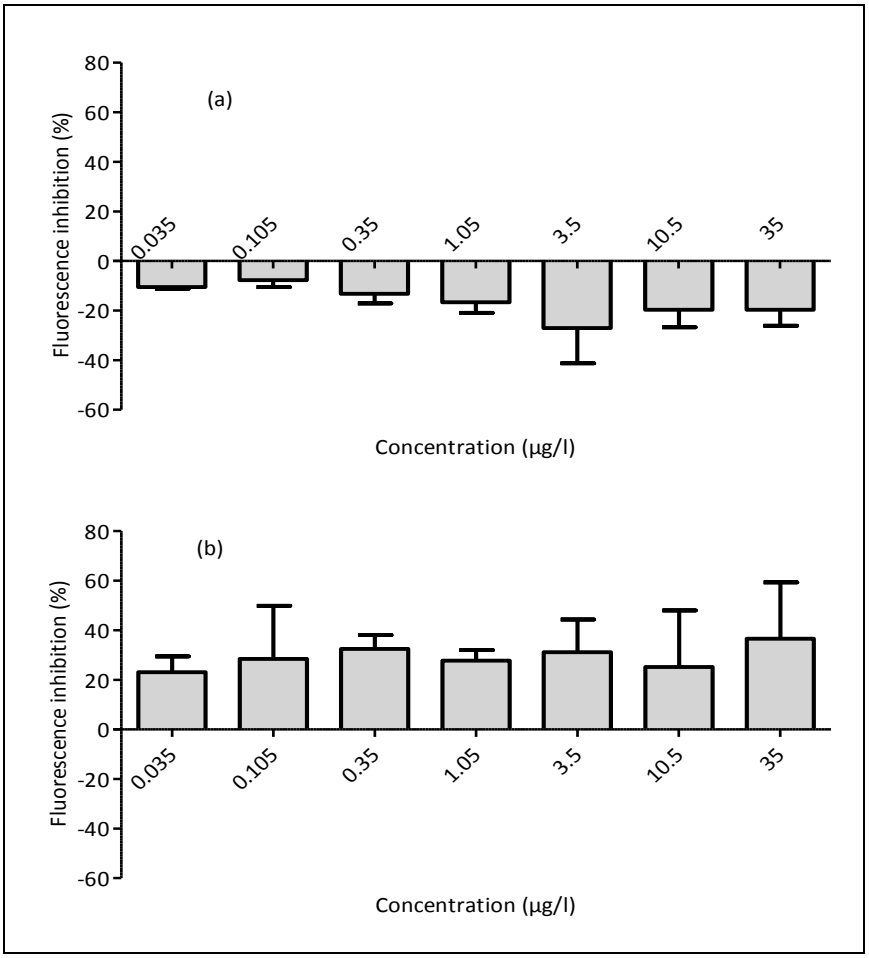


Figure 3.16: (a) Prompt fluorescence and (b) Delayed fluorescence inhibition response after 30 minutes exposure of exponentially growing algae, P. subcapitata to different concentrations of isoproturon. Standard deviation: n=4 repeated experiments

Non-parametric correlations (Spearman) analysis was performed in order to examine whether the responses of *P. subcapitata* to the four test chemicals were dependent on the mode of action of a particular test chemical or to test concentrations. There was a statistically significant correlation between isoproturon and diuron in the intensity of emitted DF (r^2=0.786, p=0.028), but no significant correlation, of the emitted DF, between 3,5-DCP and glyphosate (r^2=0.595, p=0.132). Moreover, there were no significant correlations between the mean stimulation of PF shown by diuron and isoproturon, although they have same mode of action and showed the same pattern of responses in the tests (r^2= 0.143, p>0.05, *two tailed, paired t-test*). Neither PF nor DF showed significant differences between various concentrations of test chemicals (p>0.05, 1-way ANOVA). In all four test chemicals, there were significance differences between the emitted prompt and delayed fluorescence intensity (p<0.0001). Therefore, the measured fluorescence responses shown by 3,5-DCP, glyphosate, diuron and isoproturon, on *P. subcapitata,* were independent of chemical

concentrations but dependent on the type of chemicals and their modes of action. Table 3.16 shows the pattern of responses exhibited by the four test chemicals in both DF and PF measurement.

Table 3.16: Summary of observed pattern of DF and PF after 30 minutes exposure of P. subcapitata to different concentrations of PSII inhibitors (isoproturon and diuron), and chemicals with different mode of actions (3,5-DCP and glyphosate)

Test chemical	Prompt fluorescence	Delayed fluorescence
3,5-DCP	Inhibition	Inhibition
Glyphosate	Inhibition	Stimulation
Diuron	Stimulation	Inhibition
Isoproturon	Stimulation	Inhibition

3.4.2 Identification of pattern of response for PF and DF on environmental samples

Samples, which resulted in an elevated inhibition and stimulation response in the conventional 72-hour algae growth inhibition test, were evaluated by acute algae test, to establish pattern that might be caused by sample matrix. This was based on the assumption, samples contaminated with PS II-inhibitor herbicides like diuron or isoproturon, might result in the same trend as observed in Section 3.4.1. i.e. Any sample, which showed high stimulation of PF in either chronic or acute test, would lead to a certain extent of inhibition of DF in acute test. The results are presented in Sections 3.4.2.1 and 3.4.2.2 below.

3.4.2.1 Using AAT for risk assessment of environmental/Kilombero samples

Exponentially growing *P. subcapitata* was exposed (30 minutes) to soil and sediment samples, which showed stimulation of algae growth in the conventional 72 hours algae growth inhibition test. Both PF and DF were measured, and patterns were identified. Sediment sample KSC9, which had shown highest inhibition (74%) of algae growth in the chronic (72 hours) test, resulted in 8% inhibition of prompt light emission and 23% stimulation of delayed light emission under acute tests (Fig. 3.17, Table 3.17). Sediment sample KSC1, which had shown highest stimulation (86%) in the 72 hours test, resulted in 9% and 15% inhibition of DF and PF, respectively. Therefore, the measured prompt and delayed fluorescence in sediment samples did not show trends related to presence of PS II-inhibitors. Physical properties of the samples, e.g. amount of organic nutrients, might have interfered with the emitted PF and DF.

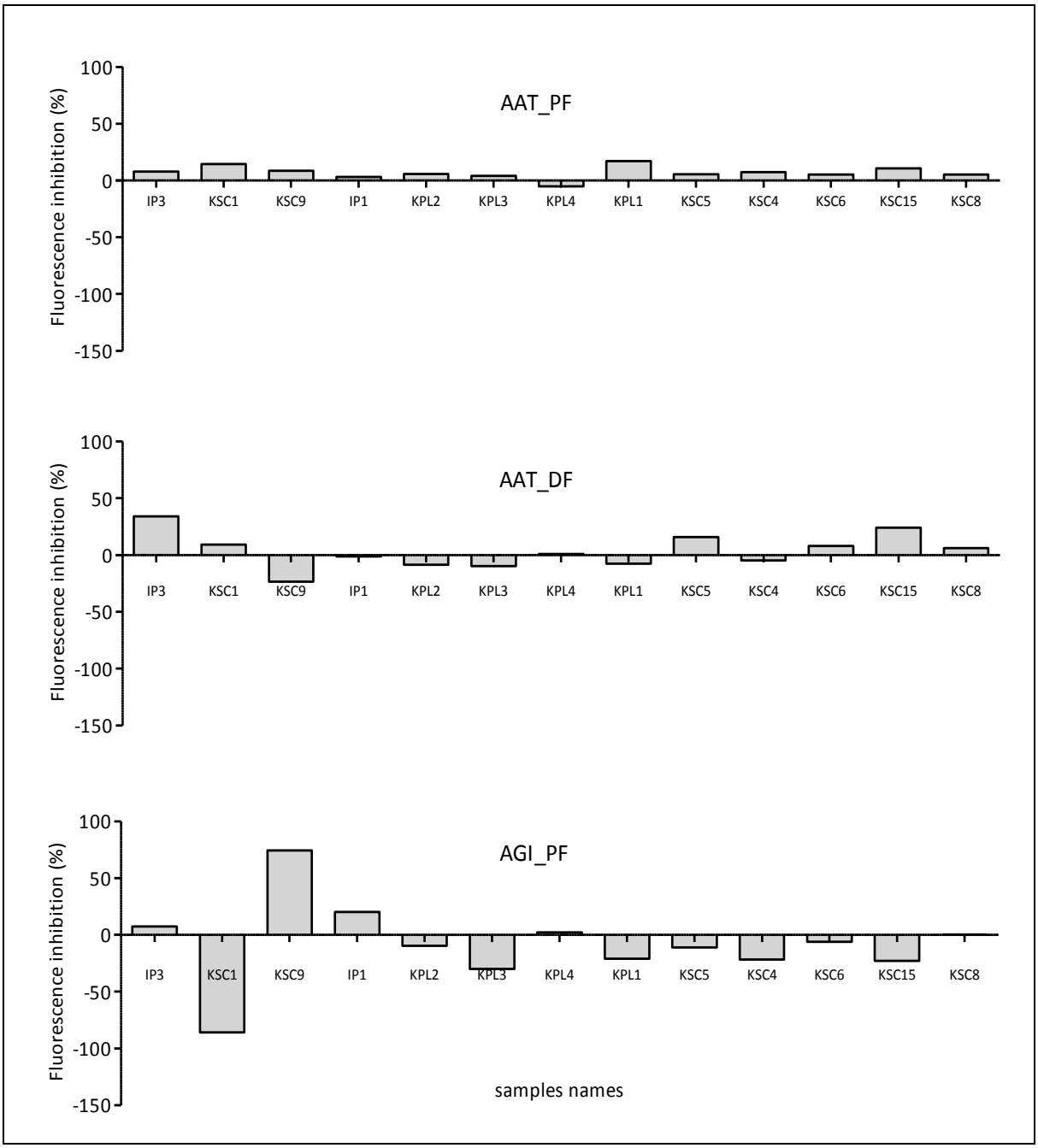


Figure 3.17: Comparison of inhibition of delayed and prompt fluorescence emitted by P. subcapitata after acute (30 minutes) and chronic exposure (72 hours) to sediment samples collected in different stations of Kilombero Valley Ramsar Site. AAT_PF and AAT_DF= Prompt and delayed fluorescence inhibition in acute algae test, AGI-PF= fluorescence inhibition in the chronic 72 hours Algae growth inhibition test.

All dry season soil samples (n=17) from Kilombero valley, resulted in stimulation of auto florescence emitted by *P. subcapitata.* However, when *P. subcapitata* was exposed to the same soil samples for 30 minutes, 3 samples out of 11 (MBS10, IP9 and KP4) showed 12% mean inhibition of DF, while the

rest of 8 samples out of 11, showed 28.8% mean stimulation of DF. For prompt fluorescence emission, one soil sample (TAC6), showed 10% stimulation of PF, while 10 samples out of 11 resulted in 13% mean inhibition of PF (Fig. 3.18). Sample MBS9, which had >100% stimulation in the chronic test, showed 22% stimulation of DF and 9% inhibition of PF in the acute test (Table 3.17 and 3.18).

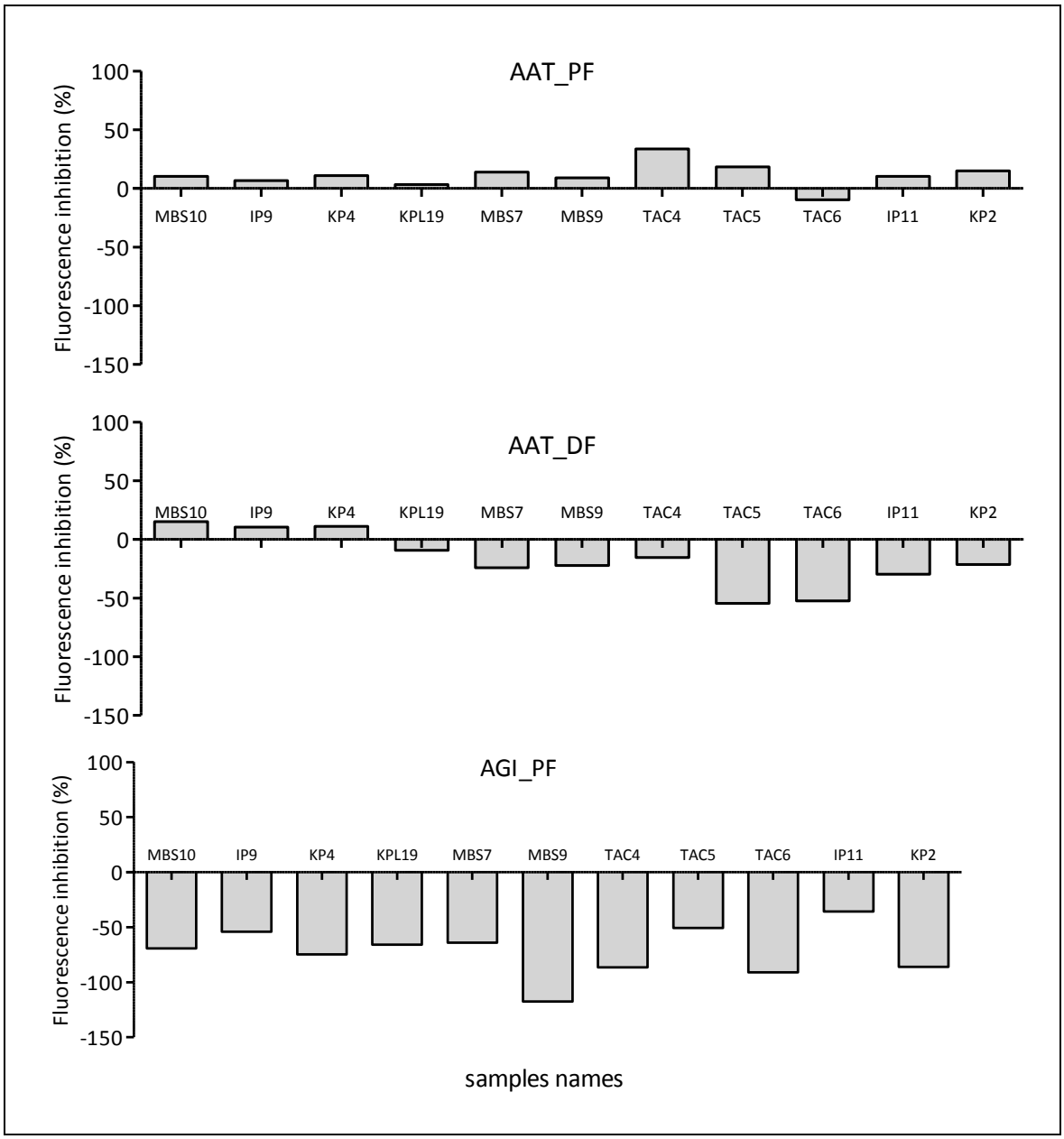


Figure 3.18: Comparison of inhibition of delayed and prompt fluorescence emitted by P. subcapitata after acute (30 minutes) and chronic exposure (72 hours) to soil samples collected in different stations of Kilombero Valley Ramsar Site. AAT_PF and AAT_DF= Prompt and delayed fluorescence inhibition in acute algae test, AGI-PF= fluorescence inhibition in the chronic 72 hours Algae growth inhibition test.

Table 3.17: Overview summary results of inhibitory or stimulatory response of P. subcapitata exposed to different sediment and soil samples. Negative values mean stimulation.

Sample Abbr.	PF inhibition (%) in AAT	DF inhibition (%) AAT	PF inhibition (%) in Chronic 72-hr AGI	detected pesticide
sediment samples				
IP3	7.90	34.06	7.39	glyphosate, AMPA
KSC1	14.53	9.25	-85.82	
KSC9	8.38	-23.29	74.71	AMPA, atrazine
IP1	3.05	-1.17	20.19	
KPL2	5.59	-8.52	-9.55	
KPL3	4.16	-9.68	-29.93	
KPL4	-5.05	0.81	2.21	
KPL1	16.94	-7.51	-21.12	
KSC5	5.49	15.73	-11.13	diuron, desmethyldiuron
KSC4	7.34	-4.84	-21.51	Diuron
KSC6	5.19	8.10	-5.89	Diuron
KSC15	10.61	24.20	-22.85	Diuron
KSC8	5.27	6.21	0.43	Diuron
soil samples				
MBS10	10.17	15.24	-69.32	Glyphosate
IP9	6.64	10.60	-54.22	Glyphosate
KP4	11.08	11.18	-74.81	glyphosate, AMPA, diuron
KPL19	3.41	-9.33	-65.87	Glyphosate
MBS7	13.98	-24.08	-64.02	
MBS9	9.01	-22.15	-117.43	
TAC4	33.70	-15.43	-86.39	
TAC5	18.24	-54.56	-50.62	
TAC6	-9.74	-52.22	-90.91	AMPA
IP11	10.17	-29.76	-35.69	glyphosate, AMPA
KP2	14.87	-21.33	-86.04	

Shading: yellow = inhibition, green = stimulation

Table 3.18 summarizes the pattern of DF and PF after 30 minutes exposure of *P. subcapitata* to sediment and soil samples of Kilombero valley. Inferences of the pattern were derived from four test chemicals 3,5-DCP, glyphosate, isoproturon and diuron, which were already tested under PF and DF measurements.

Table 3.18: Summary of the observed trend in sediment and soil samples tested under acute algae test.

Samples	DF	PF	Can be inferred to presence of chemicals with the same mode of action as:
IP3, KSC1, KSC5, KSC6, KSC15, KSC8, MBS10, IP9, and KP4	+	+	3,5-DCP
KSC9, IP1, KPL2, KPL3, KPL1, KSC4, KPL19, MBS7, MBS9, TAC4, TAC5, IP11 and KP2	-	+	Glyphosate
TAC6	-	-	Others
KPL4	+	-	phenyl urea herbicides

Legend: + inhibition, - stimulation

3.4.2.2 Spiking sediment samples with known concentration of pesticides

The objective of this experiment was to assess whether the four test chemicals in samples would lead to the same or different pattern of DF and PF compared to when the chemicals are in water only. Different concentrations of test chemicals (3,5-DCP, glyphosate, diuron and isoproturon) were added to sediment sample elutriates which had shown highest and lowest fluorescence inhibition in the conventional 72 hours algae growth inhibition test. Four sediment samples collected in Elbe river estuary during high water season were tested as shown in Table 3.19. Selection of samples were governed by the following criteria: (i) freshness (1 - 2 weeks old) by the time these experiments were conducted; (ii) *P. subcapitata* ecotoxicity data which were available; and (iii) if, among other samples of the same batch, the selected samples had shown either <10% or >80% inhibition of the emitted auto-fluorescence. Therefore, by considering the fact that the samples had shown extreme high and low responses in chronic tests, it would be easy to notice any interference of their matrices in the acute tests.

Table 3.19: Names of sediment samples spiked with test chemicals

	Given name	Chronic AGI-results	samples origin
i.	SL-0	Low inhibition responses (<10%)	*Elbe River estuary, high water season*
ii.	SH-1	High inhibition responses (>80%)	
iii.	SH-2		
iv.	SH-3		

Table 3.20: Summary of PF and DF inhibition results upon addition of test chemicals to samples

Chemical	Concentration	% Inhibition of emitted auto-fluorescence					
		in sterile water		**in sample SL-0**		**in sample SH-2**	
		PF	**DF**	**PF**	**DF**	**PF**	**DF**
3,5-DCP (µg/L)	0.035	-13.9	-6.6	-30.9*	-17.3	-35.8*	-17.7
	0.105	-23.5	-43.8	-35.8	-47.1	-36.6	-15
	0.35	-25.3	-7.3	-25.2	-18.6	-26.2	-52.3
	1.05	-27.4	-39.8	-33.9	-60.8	-34.4	-17.2
	3.5	-31.6	-15.8	-30.6	-63.3	-36.7	-29.2
	10.5	-33.4	-29.7	-27.8	9.0	-34.1	17.2
	35	-33.2	1.7	-31.4	-35.5	-33.7	11.7
		in sterile water		**in sample SL-0**		**in sample SH-1**	
		PF	**DF**	**PF**	**DF**	**PF**	**DF**
glyphosate (mg/L)	0.6	-34.6	-23.9	-20.1	-8.4	-29.0	-13.4
	0.7	-39.4	-17.0	-15.1	-27.7	-34.0*	-17.1
	0.84	-35.8	-29.3	-21.3	-5.0*	-32.9	-25.3
	1.05	-37.3	-64.1	-28.1	-46.6	-32.7	-7.6**
	1.4	-36.9	-66.6	-24.9	-21.2*	-34.9	3.3**
	2.1	-40.0	-61.1	-30.3	-38.4	-34.7	-13.5**
	4.2	-36.5	-50.2	-36.8	-64	-33.5	-9.4**
		in sterile water		**in sample SH-2**		**in sample SH-3**	
		PF	**DF**	**PF**	**DF**	**PF**	**DF**
isoproturon (µg/L)	0.035	-48.6	-9.8	-51	10.5*	-38.3	10.6
	0.105	-59.8	-41.7	-54.6	6.4**	-55.3	7.1**
	0.35	-58.6	-9	-56.8	-10.2	-73.3	16.5
	1.05	-62.9	63.5	-58.4	8.0**	-62.8	2.5***
	3.5	-69.7	53.1	-68.6	-5.5**	-71.1	39.5
	1.05	-70.5	30.5	-69	-16.9*	-68.4	54.6
	35	-72.5	39.0	-63.3	11.3	-67.5	79.9*
		in sterile water		**in sample SH-2**		**in sample SH-3**	
		PF	**DF**	**PF**	**DF**	**PF**	**DF**
diuron (µg/L)	0.035	-70.0	-9.77	-46.2*	51.47**	-48.8**	88.51***
	0.105	-68.1	-41.72	-50.7	4.18**	-57.0	47.81**
	0.35	-66.0	-8.99	-52.3	22.75*	-50.5	34.67**
	1.05	-71.6	63.5	-58.4	-20.56**	-61.6	64.15
	3.5	-79.4	53.14	-71.8	40.27	-59.0	79.05
	1.05	-81.4	30.48	-59.3	26.7	-67.4	34.91
	35	-91.3	39.05	-68.4*	83.34**	-56.7*	81.46*

Shades yellow=inhibition, green= stimulation. *The inhibition or stimulation response in samples is significant compared to a chemical in sterile water at a level of * p ≤ 0.05; **p≤ 0.01; ***p ≤ 0.001. (1-way ANOVA, post-hoc- Dunnett`s multiple comparison tests).*

With an exception of 35 µg/L, which showed 1.7% inhibition in DF, 3,5-DCP showed a general pattern of stimulation of both PF and DF at all other concentrations while in water but not in sample elutriates (Table 3.20). Concentrations lower than 10.5 µg/L 3,5-DCP (in samples SL-0, SH-2) showed stimulation of both PF and DF, while highest concentration 35 µg/L 3,5-DCP (in samples SH-2) and 10.5 µg/L 3,5-DCP (in SL-0, SH-2), showed inhibition of DF. However, only the lowest concentration (0.035 µg/L) of 3,5-DCP (in samples SL-0 and SH-2), showed significantly higher stimulation of emitted prompt light when compared to same concentration of 3,5-DCP in sterile water (p>0.05, Table 3.20). For glyphosate, with an exception of 1.4 mg/L (in sample SH-1), which showed inhibition of DF, all concentrations of glyphosate in water and in samples (SL-0, SH-1) showed stimulation of both PF and DF. There was a significant differences in the DF stimulation exhibited by glyphosate in samples as compared to when in water ($p<0.05$, exact-levels are shown in Table 3.20).

All concentrations of diuron and isoproturon showed stimulation of PF while in water, and inhibition of DF while in samples (SH-3 only). Both diuron and isoproturon concentrations higher than 1.05 µg/L (in water), showed inhibition of DF, while concentrations lower than 1.05g/l (in water), exhibited stimulation of DF. Isoproturon in sample SH-2 showed both stimulation and inhibition of DF at various concentrations, while, with an exception of 1.05 µg/L that showed stimulation, all concentrations of isoproturon (in samples SH-2), showed inhibition of DF, as shown in Table 3.20. A significant reduction of prompt fluorescence emitted by algae was observed at 0.035 µg/L of diuron in samples SH-2 and SH-3, and at 35 µg/L of diuron in sample SH-3 (p<0.05, 1-way ANOVA).

3.4.3 Relationship between algae auto fluorescence and cell density

Exponentially growing *P. subcapitata* were exposed for 72-hour to different concentrations of the four test chemicals (3,5-DCP, glyphosate, diuron and isoproturon). Inhibition of algae auto fluorescence and cell density of the test matrices was calculated in comparison to negative controls.

3.4.3.1 Fluorescence intensity and cell density in 72 hours AGI

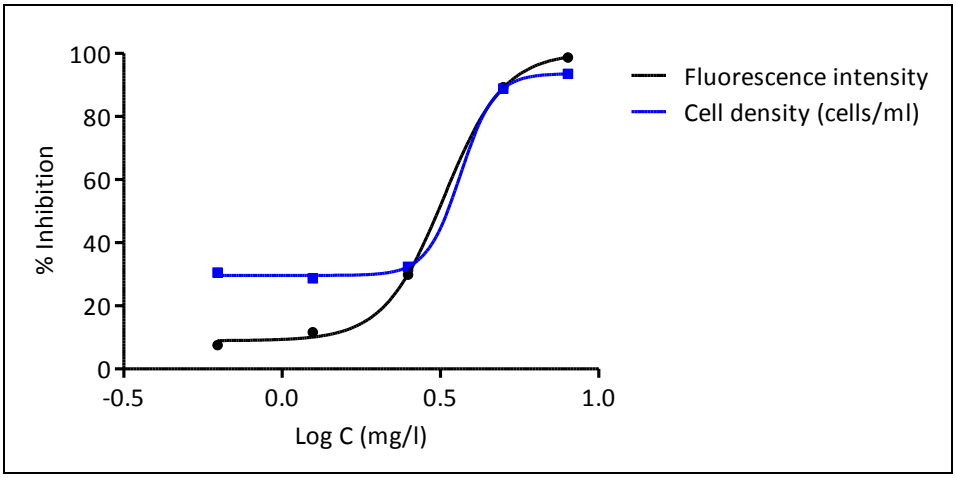


Figure 3.19: Dose-response curve of fluorescence intensity and cell density inhibition after 72 hours exposure of P. subcapitata to 3,5-dichlorophenol.

From Fig. 3.19, algae cell density and its emitted auto-fluorescence intensity decreased with increased concentration of 3,5-DCP in the test matrix. The highest concentration 8 mg/L of 3,5-DCP resulted in 99% and 94% fluorescence and cell density inhibition, respectively. While the lowest concentration 0.625 mg/L (3,5-DCP) resulted in 7.5% and 30.5% inhibition of fluorescence and cell density, respectively.

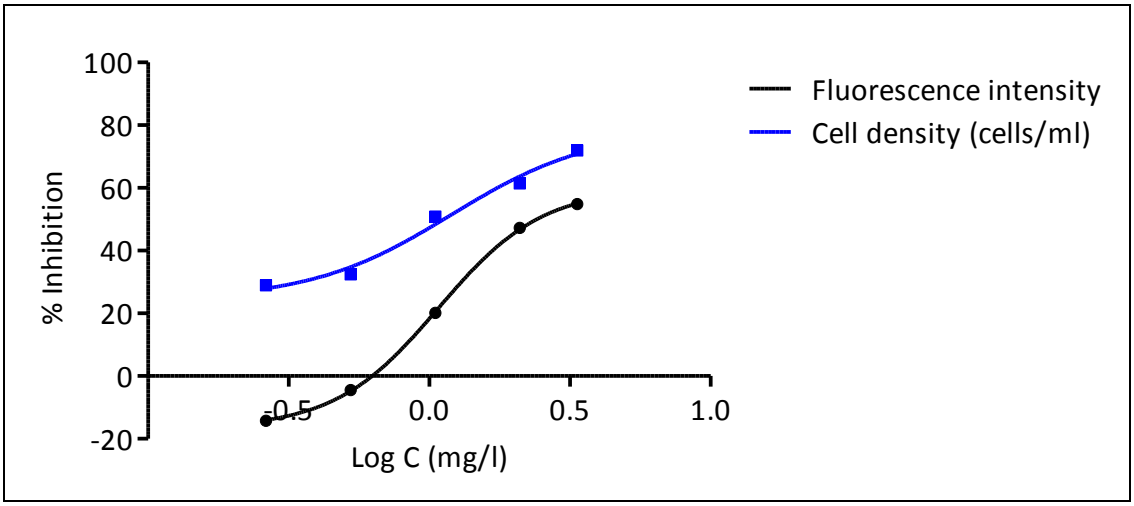


Figure 3.20: Dose-response curve of fluorescence intensity and cell density inhibition after 72 hours exposure of P. subcapitata to glyphosate

Fig. 3.20 shows that cell density and the intensity of emitted auto-fluorescence by *P. subcapitata,* decreased with increased concentration of glyphosate in the test matrix. 14% and 4% stimulation of

fluorescence intensity was shown by 0.2625 mg/L and 0.525 mg/L of glyphosate, respectively. Highest concentration of glyphosate (3.36 mg/L) resulted 72% and 56% inhibition of cell density and fluorescence intensity, respectively. Generally, the inhibition of cell density was at higher magnitude of the steep hill slope than fluorescence intensity.

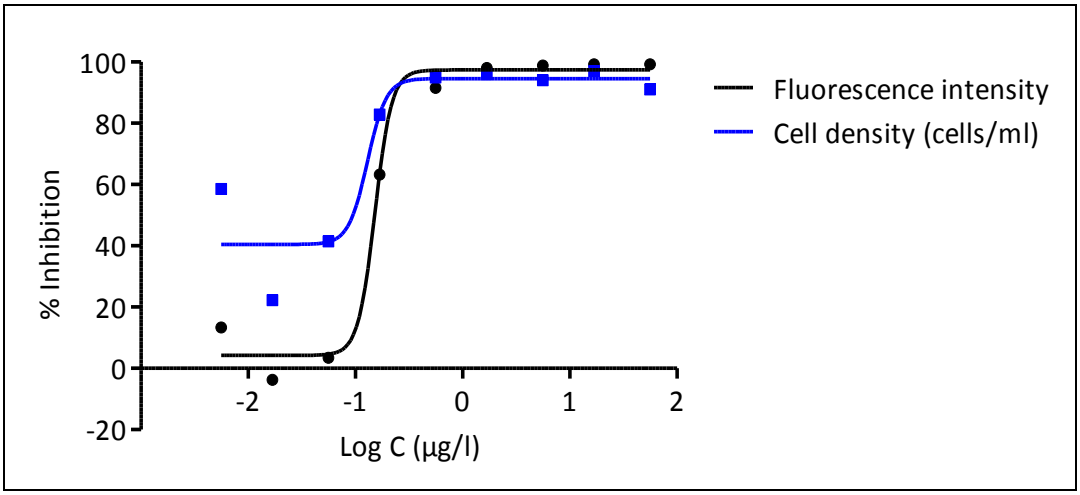


Figure 3.21: Dose-response curve of fluorescence intensity and cell density inhibition after 72 hours exposure of P. subcapitata to diuron.

Figure 3.21 indicates that diuron concentration higher than 0.560 µg/L, resulted in 97% and 95% mean inhibition of fluorescence intensity and cell density, respectively. While diuron concentrations lower than 0.168 µg/L showed 27% and 51% mean inhibition of fluorescence intensity and cell density, respectively. There was a 3% stimulation of fluorescence intensity at 0.056 µg/L of diuron. In general, fluorescence intensity and cell density inhibition increased with increased concentration of diuron in the test matrices.

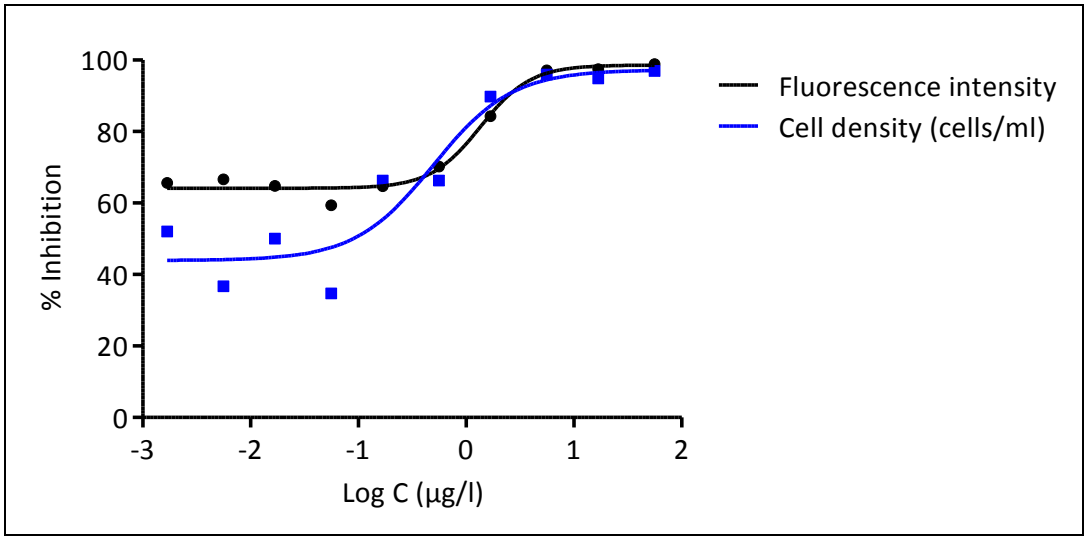


Figure 3.22: Dose-response curve of fluorescence intensity and cell density inhibition after 72 hours exposure of P. subcapitata to isoproturon.

In Fig. 3.22, inhibition of fluorescence intensity and cell density decreased with increased concentrations of isoproturon. Therefore, isoproturon concentrations higher than 0.056µg/L showed 80% and 68% inhibition of fluorescence intensity and cell density, respectively. Minimum inhibition of fluorescence intensity and cell density were 59% and 35% respectively by 0.056 µg/L of isoproturon.

Table 3.21: Summary of IC_{50} range values of fluorescence intensity and cell density inhibition after exposure of P. subcapitata to four test chemicals (at 95% confidence interval, in the brackets are best -fit values)

Test chemical	**IC_{50}**	
	Fluorescence intensity inhibition	**Cell density inhibition**
3,5-DCP	1.961 to 5.389 (3.251) mg/L	2.077 to 6.457 (3.662) mg/L
glyphosate	0.7780 to 1.494 (1.078) mg/L	(1.199) mg/L
diuron	0.06411 to 0.3614 (0.1522) µg/L	0.01387 to 1.213 (0.1297) µg/L
isoproturon	(>50% inhibition in all concentrations)	0.1323 to 1.866(0.4969) µg/L

Table 3.21 indicates the summary of calculated IC_{50} values from the cell density and fluorescence intensity inhibition. The IC_{50} values for the two parameters are almost of the same magnitude, except for isoproturon where inhibition of fluorescence intensity was higher than 50% in all concentrations.

3.4.3.2 Emitted prompt fluorescence per cell in 72 hours AGI

The objective of calculating prompt fluorescence per cell, was to assess whether the measured stimulation due to auto fluorescence was correlated to actual number of algae cells in the matrix. Algae cells were counted in the conventional 72 hours-algae growth inhibition test, because it involves multiple cell generation, and thus easy to compute the cell density after exposure to test chemicals. In order to evaluate the potential impact of varying concentration of contaminants, different concentrations of test chemicals (3,5-DCP, glyphosate, diuron and isoproturon) were used.

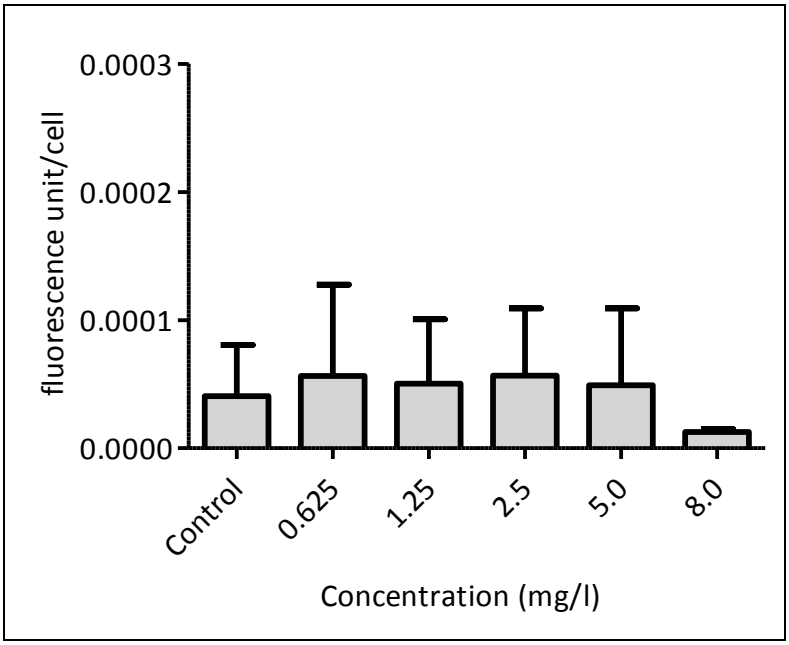


Figure 3.23: Overview of relative units of emitted prompt fluorescence per cell after 72 hours exposure of P. subcapitata to 3,5-DCP. Standard deviation: n=3 replicates.

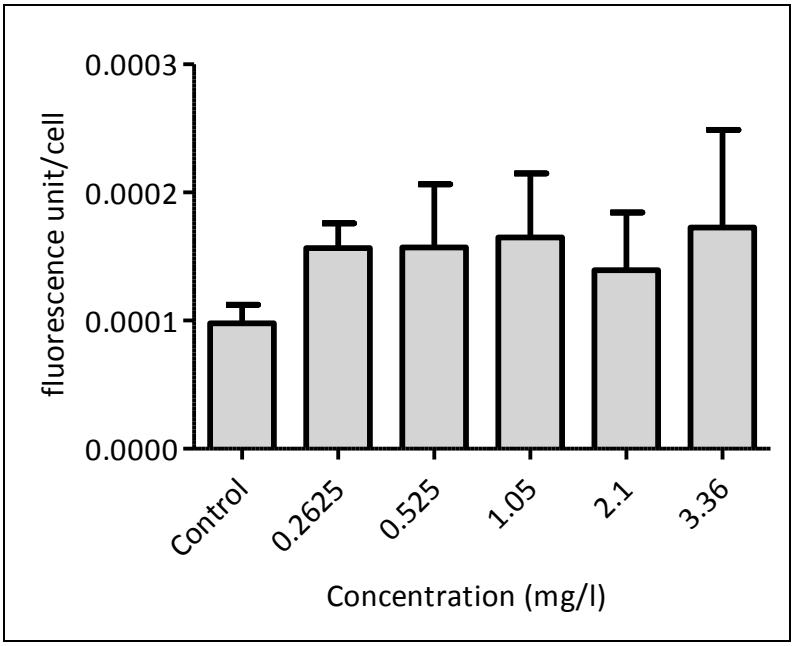


Figure 3.24: Overview of relative units of emitted prompt fluorescence per cell after 72 hours exposure of P. subcapitata to glyphosate-Roundup. Standard deviation: n=3 replicates.

The highest concentration of 3,5-DCP (8 mg/L) resulted in lowest fluorescence unit per cell ($1.25x10^{-5}$), which indicates high inhibition of fluorescence (Fig. 3.23). The observed emitted fluorescence per cell was lower in the controls than in all test-concentrations of glyphosate (Fig.3.24), which indicates stimulation of fluorescence. Paired *t* test revealed significant increase of mean fluorescence unit per cell in all concentrations of glyphosate as opposed to 3,5-DCP concentrations (p=0.0006). However, neither 3,5-DCP nor glyphosate, showed significant differences of emitted fluorescence per cell between different concentrations (p>0.05, 1-way ANOVA, *Post-hoc*: Dunn´s multiple comparison test).

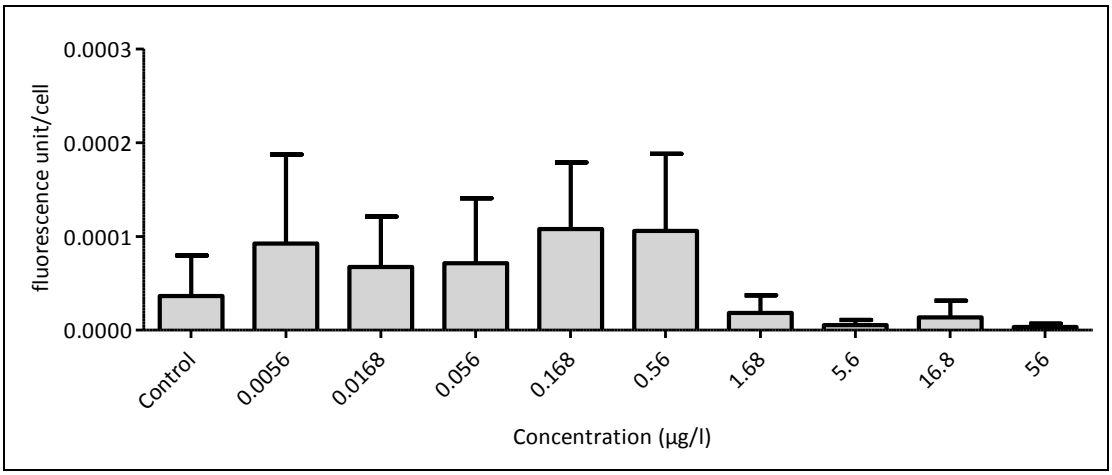


Figure 3.25: Overview of relative units of emitted prompt fluorescence per cell, after 72 hours exposure of P. subcapitata to various concentrations of diuron. Standard deviation: n=3 replicates.

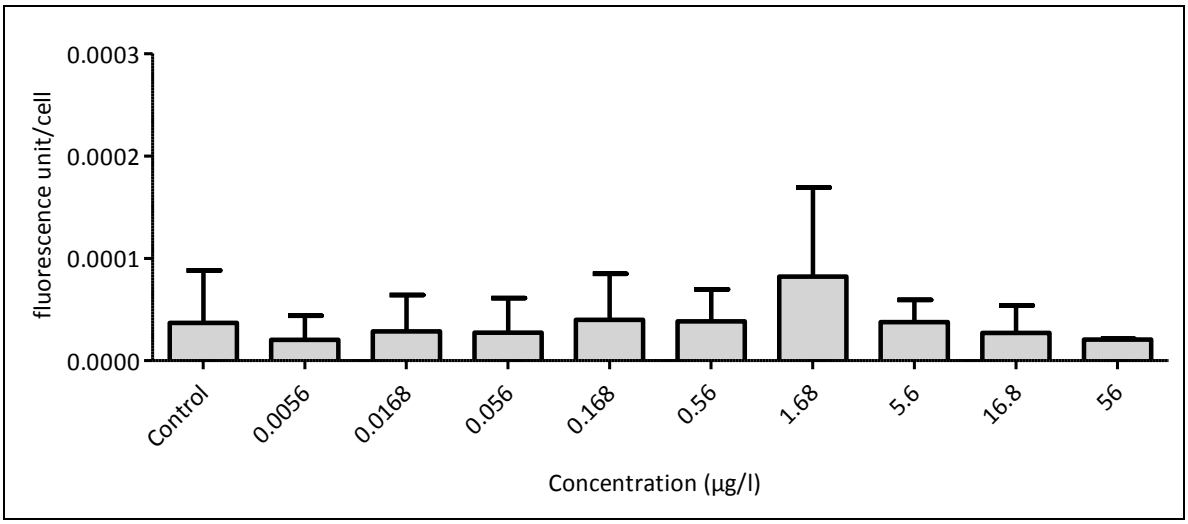


Figure 3.26: Overview of relative units of emitted prompt fluorescence per cell, after 72 hours exposure of P. subcapitata to different concentrations of isoproturon. Standard deviation: n=3 replicates.

Diuron concentrations, from 0.0056 µg/L to 0.56 µg/L, showed two-fold higher fluorescence per cell, than the same concentrations of isoproturon (Figs. 3.25 and 3.26). In contrary, higher concentrations of isoproturon, from 1.68 µg/L to 56 µg/L, showed two-fold higher fluorescence unit per cell than the same concentrations of diuron. However, neither diuron nor isoproturon had significant differences of fluorescence per cell shown by different concentrations (p>0.05, 1-way ANOVA, *Post-hoc*: Dunn´s multiple comparison test). In general, the fluorescence per cell at different concentrations of either diuron or isoproturon, differ significantly from that of glyphosate concentrations (p=0.0022) but not with 3,5-DCP (p>0.05, 1-way ANOVA, *post-hoc:* Dunn´s multiple comparison test).

3.4.3.3 Elevated nutrient investigation

Exponentially growing *P. subcapitata* was exposed to different concentrations of nutrient sources in an elevated concentrations while maintaining other essential constituents nutrients for algal growth in the test medium. The enrichment factors of elevated concentrations of DIN-algae growth nutrients used in this experiment are shown in Table 3.22. Inhibition of growth rate of algae on every concentration compared to negative controls was calculated from the emitted auto-fluorescence after 72 hours exposure of *P. subcapitata* to the excess nutrients conditions.

Table 3.22: Summary of different nutrients concentrations for testing impacts of excess nutrients on P. subcapitata growth rate

Nutrient source	Substance	Test concentrations				
P-source (mg/L)	KH_2PO_4	1.6	8	16	32	80
N-source (mg/L)	NH_4CL	16	80	160	320	800
C-source (mg/L)	Peptone	40	200	400	800	2000
3,5-dichlorophenol (mg/L)	Positive control	0.625	1.25	2.5	5.0	8.0
		Enrichment factors				
Excess micronutrients	DIN-STD1	1.25	2.5	5	10	16
Excess trace elements	DIN-STD3					
Excess Fe^{2+} (mg/L)	DIN-STD2					
Vitamins(mg/L)	vitamin B1&B12					
Sterile water	Negative Control					

Lowest concentration (16 mg/L) of the Nitrogen source (NH_4^+-N) in form of NH_4CL resulted in 3% inhibition while concentration greater than 80 mg/L resulted in critical inhibition between 44% and 99% (Fig. 3.27). Only 5% algae growth stimulation was shown by $PO4^{2-}$ concentration below 8 mg/L while higher concentrations than that increasingly inhibited *P. subcapitata* to 57% (maximum) at highest concentration (80 mg/L). Conversely, presence of extra carbon with concentration less than 400 mg/L resulted in 45% mean stimulation of algae growth. All concentrations of vitamins B1&B12 in the test medium resulted in stimulation of algae growth.

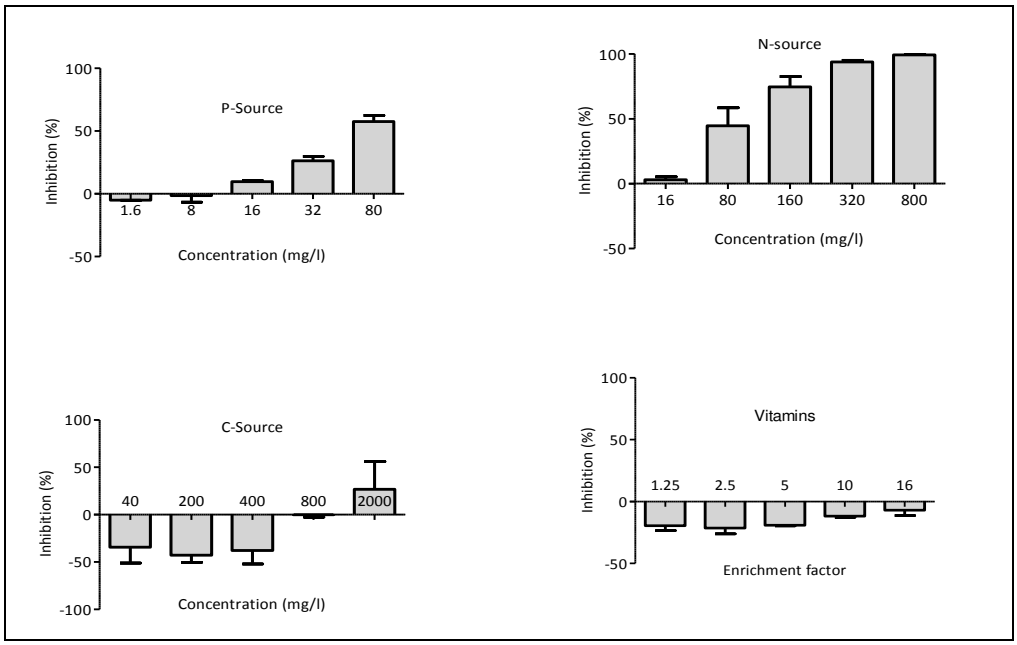


Figure 3.27: Inhibition of growth rate of P. subcapitata by elevated concentration of nutrients. P-source = KH_2PO_4, N-source = NH_4Cl, C-source = peptone, Vitamins = B1 & B12 complex. Standard deviations: n=6 replicates

Excess micronutrients such as Ca^{2+}, K^{+}, Mg^{2+} and Cl (DIN standard 1), inhibited *P. subcapitata* growth rate from 33% to 95% in all concentrations of enrichment factor higher than 2.5. The lowest concentration (enrichment factor 1.25) showed 2.6% stimulation (Fig. 3.28). Increasing the amount of trace elements such as Zn^{2+}, Cu^{2+}, Mo^{6+}, Co^{2+}, and Mn^{2+} (DIN standard 3) in the test matrix by factors of 10 and 16, led to inhibition of 33% and 93%, respectively. In the contrary, lower enrichment factors, 1.25, 2.5 and 5 of trace elements (DIN Standard 3) in the matrix resulted in 8%, 12% and 17% stimulation of algae growth rate, respectively (Fig. 3.28). The mixtures of carbon source (peptone) and vitamins (B_1&B_{12}) at all enrichment factors, as opposed to individual nutrient source, enhanced stimulation of *P. subcapitata*. For instance, 47% inhibition was shown by highest concentrations of carbon-source (2000 mg/L peptone) while it stands alone but when the same peptone concentration is mixed with vitamins, it resulted in 24% stimulation. Lower concentrations of DIN standard 2 (Fe^{2+}) i.e. enrichment factors ≤ 10, stimulated algae growth rate between 8% and 13% while highest concentration with enrichment factor of 16 resulted in 21% inhibition.

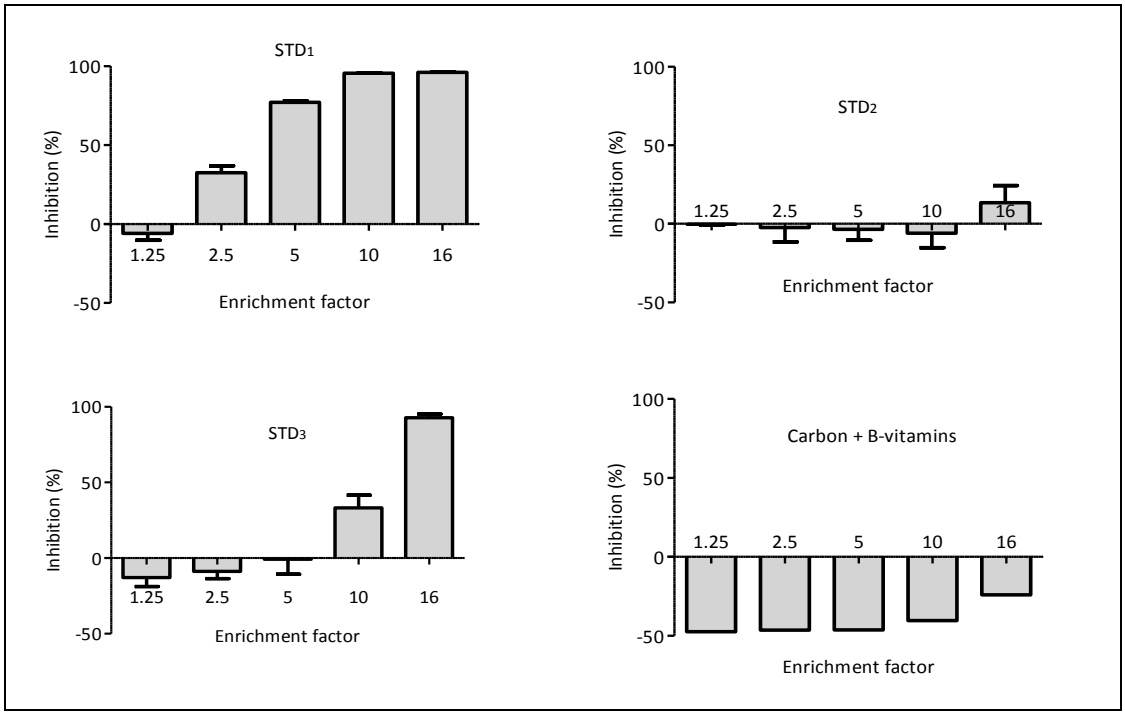


Figure 3.28: Inhibition of algae, growth rate after 72-hours exposure of P. subcapitata to elevated concentration of nutrients. STD1= excess micronutrients, STD2=excess Fe^{2+}, STD3= excess trace elements. Standard deviation: n=6 replicates.

3.5 Yeast, *S. cerevisiae* bioassay

The aim of this test was to assess the impact of environmental contaminants, such as fungicides that are widely used in agricultural fields, on fungi *S. cerevisiae.* The test procedure and conditions such as resazurin concentration, incubation period of yeast cells with test matrices and with resazurin, were developed and optimized in our laboratory. Results for the bioassay development and optimization are presented in Section 3.5.1.1. The optimized conditions and procedures were used to assess impact of contaminated sediment, soil and water samples collected from Kilombero valley sugarcane and rice plantations on *S. cerevisiae,* (results are summarized in Section 3.5.1.3).

3.5.1 Development of a *S. cerevisiae* bioassay using resazurin based on Fai and Grant (2009b)

3.5.1.1 Optimization of resazurin concentration and exposure time

Yeast, *S. cerevisiae* were exposed to increasingly varying concentrations of copper (II) sulphate. In order to optimize conditions that could result into a potential effect on the yeast metabolic activities, different incubation periods (1 to 4 hours) of fungicide with resazurin concentrations were tested. Additionally, in order to establish the ideal concentrations that would produce feasible resorufin fluorescence intensity, after exposure of yeast cells to test chemicals, different concentrations of resazurin were tested. This is because very pale pink-resorufin fluorescence was produced in the earlier experiments, where resazurin concentrations lower than those used in this study were used. The pale pink-resorufin turned shortly to colorless (hydro-resorufin) while measurements were underway. Percentage inhibition of resorufin fluorescence intensity compared to negative controls was used to evaluate the responses of yeast cells to $CuSO_4$. Table 3.23 summarizes the resorufin fluorescence inhibition resulted from different exposure time to copper sulphate and different range of resazurin test concentrations.

1 hour: One hour incubation of yeast cells with low concentrations of copper sulphate (<1.5 mg/L) did not cause a significant inhibition of fluorescence in all resazurin concentrations (p>0.05, 1-way ANOVA, Table 3.23). Highest concentration of copper sulphate (5 mg/L) showed less than 30% resorufin inhibition after one hour incubation irrespective of initial resazurin concentration.

2.5 hours: After 2.5 hours of exposure, highest concentration (5 mg/L) of copper sulphate showed 50 ± 4% resorufin fluorescence inhibition at all concentrations of resazurin. Lower concentrations (<5 mg/L) of copper sulphate showed inhibition less than 35% irrespective of resazurin concentration (Table 3.23).

4 hours: After 4 hours of exposure, 5 mg/L of copper sulphate showed inhibitions of 51% and 50% at resazurin concentrations of 10 mg/L and 15 mg/L respectively (Table 3.23). Lower concentrations of copper sulphate had no significant impact on the metabolic activity of *S. cerevisiae* at all resazurin concentrations.

Table 3.23: A summary of the percentage dehydrogenase activity of S. cerevisiae under different resazurin concentration and copper sulphate

Incubation period of *S. cerevisiae* with $CuSO_4$	Conc. $CuSO_4$ (mg/L)	Resazurin concentration and resulting % inhibition				
		2 mg/L	5 mg/L	10 mg/L	15 mg/L	20 mg/L
1 hour	0.01	4.6	6.5	-2.6	0.7	-2.1
	0.02	8.5	7.0	0.8	3.1	0.6
	0.05	20.7	13.4	2.8	6.3	7.7
	0.15	18.6	16.7	7.4	10.6	12.6
	0.50	8.9	12.9	8.1	12.0	13.6
	1.50	11.0	13.8	13.3	17.9	20.7
	5.00	22.9	26.6	23.5	29.8	29.7
2.5 hours	0.01	9.2	5.6	3.8	2.2	3.3
	0.02	13.4	11.4	9.3	6.8	8.1
	0.05	25.6	21.0	18.5	16.1	17.9
	0.15	30.0	26.3	25.9	22.3	24.6
	0.50	32.2	29.9	28.8	26.6	26.7
	1.50	36.0	37.5	38.3	33.3	33.4
	5.00	52.3	49.7	47.8	46.5	45.8
4 hours	0.01	2.6	2.5	2.5	-2.0	-4.6
	0.02	4.9	6.0	6.3	4.3	3.0
	0.05	10.6	11.8	16.8	16.9	12.2
	0.15	15.8	18.9	24.2	25.5	21.6
	0.50	20.8	23.4	31.3	31.4	22.0
	1.50	29.4	32.7	38.9	40.8	26.0
	5.00	44.9	47.2	51.4	50.2	33.5

shaded= % resorufin inhibition $\sim 50 \pm 5$

Therefore, highest concentration of copper sulphate (5 mg/L) in all resazurin concentrations showed higher inhibition response than in the lower concentrations (≤1.5 mg/L). However, with an exception of 4 hours exposure (20 mg/L of resazurin), which showed 33.5% inhibitions, highest inhibitions of 50 ± 5% occurred during 2.5 and 4 hours of exposure of yeast cells to 5 mg/L of

copper sulphate at all resazurin test concentrations (Table 3.23). Therefore, 4 hours exposure time of yeast cells with other fungicides (prochloraz and thiabendazole) was chosen in order to provide sufficient time for yeast cells to acclimatize and grow in the micro-well plates.

3.5.1.2 Optimization of positive control

The results in the optimization of resazurin and incubation period of copper sulphate were evaluated. Since 10 mg/L concentration of resazurin had shown a consistently an increasing trend in almost all incubation periods, as summarized in Table 3.23, it was favored for experiments with other selected fungicides at different incubation periods in order to establish a suitable positive control for *S. cerevisae* bioassay.

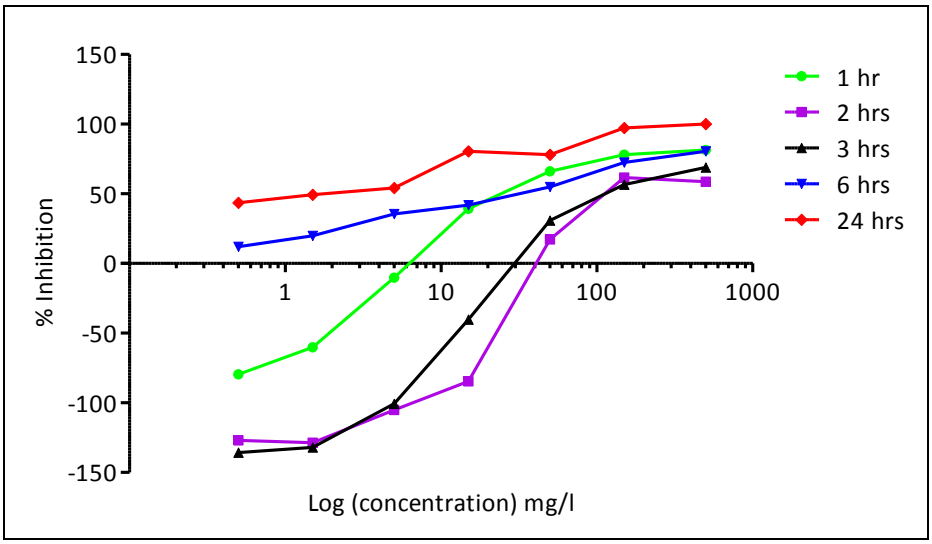


Figure 3.29: Percentage of dehydrogenase inhibition measured by resorufin fluorescence after different hours of exposure of S. cerevisiae to different concentrations (in a logarithmic scale) of copper sulphate. Resazurin concentration used 10 mg/L.

Response of *S. cerevisiae* to copper sulphate test concentrations differed significantly between lengths of exposure (*p<0.0001, 1-way ANOVA).* For instance, incubation period of 1 to 3 hours, showed stimulation in the $CuSO_4$ concentrations lower than 15 mg/L, while concentrations higher than 50 mg/L, showed gradual increase of inhibition (Fig. 3.29). There was no stimulation observed by different concentrations of copper sulphate in extended exposure periods (>6 hours). Multiple comparison tests revealed a significant gradual increase of resorufin fluorescent inhibition from the lowest (0.5 mg/L) to highest (500 mg/L) concentrations of $CuSO_4$ incubated with yeast cells for 6 and 24 hours ($p<0.05$)

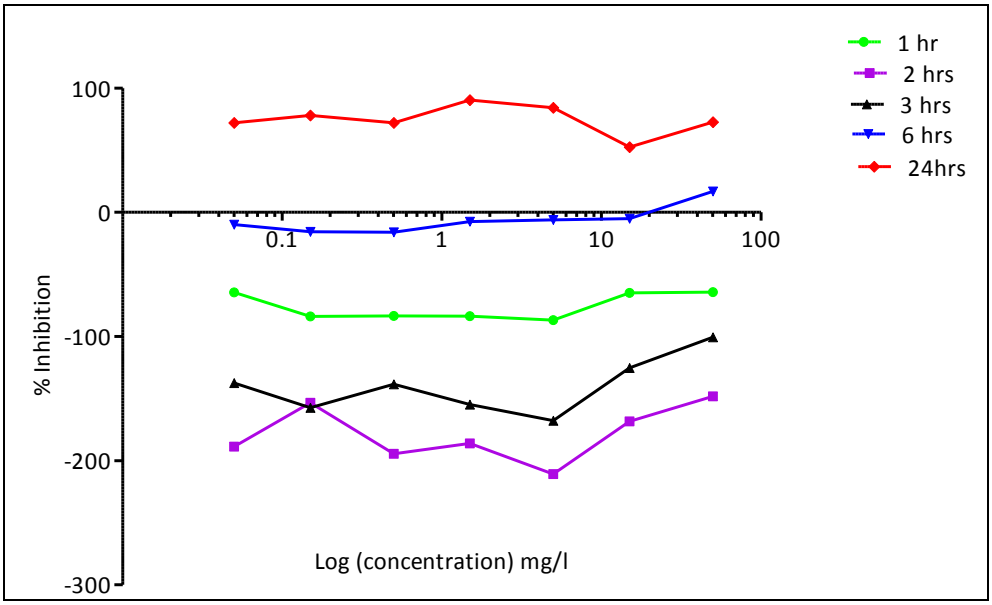


Figure 3.30: Percentage of dehydrogenase inhibition measured by resorufin fluorescence after different hours of exposure of S. cerevisiae to different concentrations (in a logarithmic scale) of thiabendazole. Resazurin concentration used 10 mg/L.

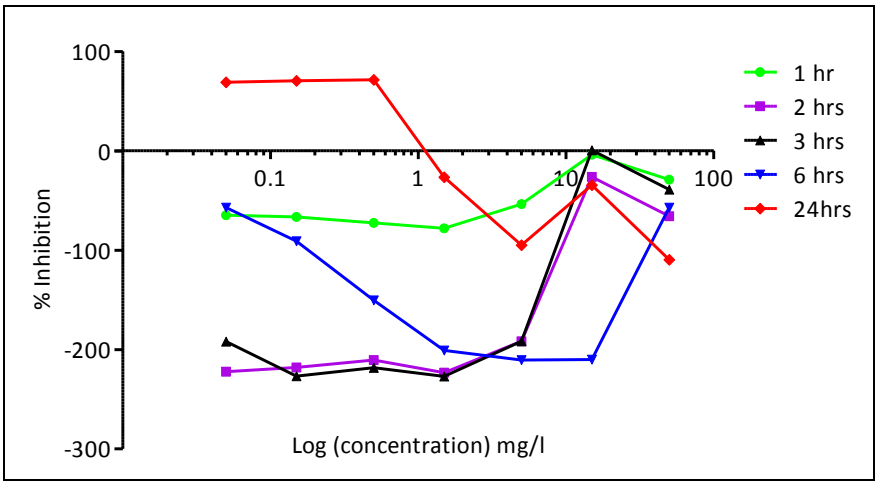


Figure 3.31: Percentage of dehydrogenase inhibition measured by resorufin fluorescence after different hours of exposure of S. cerevisiae to different concentrations (in a logarithmic scale) of prochloraz. Resazurin concentration used 10 mg/L.

Figures 3.30 and 3.31 indicate that regardless of test concentration used, shorter incubation period of yeast cells (up to 6 hours) with organic fungicides, thiabendazole and prochloraz, resulted in a significant resorufin fluorescence stimulations (p<0.0001, *1-way ANOVA*). However, 24 hours exposure of yeast cells to all concentration of thiabendazole showed 74.5% mean inhibition while concentration of prochloraz lower than 1.5 mg/L resulted to 70.3% mean inhibition of resorufin fluorescence intensity. The stimulation trend (24-hour exposure), increased significantly, from prochloraz concentration higher than 1.5 mg/L (p<0.05, Fig. 3.31). In general, *S. cerevisiae* was

stimulated with different concentrations of fungicides prochloraz and thiabendazole in the test matrices in all incubation periods less than 24 hours (Table 3.24).

Table 3.24: Summary of percentage inhibition of S. cerevisiae dehydrogenase activity by three fungicides in different incubation periods, using 10 mg/L of resazurin concentration

Concentration (mg/L)	Incubation period of yeast with test chemicals (hours)				
	1	**2**	**3**	**6**	**24**
Copper sulphate					
0.5	-79.5	-127.0	-135.8	12.0	43.6
1.5	-60.1	-128.5	-132.0	19.8	49.3
5	-10.1	-105.2	-100.6	35.6	54.3
15	39.3	-84.7	-40.1	41.9	80.5
50	66.2	17.3	30.9	54.9	78.1
150	78.1	61.6	56.7	72.5	97.
500	81.5	58.7	68.9	80.6	100.0
Thiabendazole					
0.05	-64.5	-188.7	-137.5	-9.9	72.0
0.15	-83.9	-153.5	-157.5	-15.8	77.9
0.5	-83.4	-194.4	-138.4	-16.1	72.0
1.5	-83.6	-186.0	-154.8	-7.6	90.4
5	-86.9	-210.8	-167.8	-6.2	84.2
15	-65.0	-168.4	-125.3	-5.2	52.4
50	-64.4	-148.1	-100.5	16.8	72.6
Prochloraz					
0.05	-64.7	-222.2	-191.9	-57.2	69.0
0.15	-66.6	-218.1	-226.8	-90.9	70.5
0.5	-72.5	-210.6	-218.3	-150.5	71.5
1.5	-78.0	-223.1	-226.9	-201.0	-26.6
5	-53.5	-191.6	-191.6	-210.5	-94.9
15	-3.8	-26.3	0.7	-210.2	-34.4
50	-28.9	-65.9	-38.9	-57.0	-109.6

Shaded: yellow = inhibition, green = stimulation

Table 3.25 summarizes the optimized conditions for the developed yeast, *S. cerevisiae* bioassay. The rest of the parameters tested during optimization did not show a steady trend.

Table 3.25: Optimized parameters and necessary condition for a proposed S. cerevisae bioassay

	Optimized parameter	Condition
1	Test matrices incubation periods with samples / with fungicides	4 hours
2	Resazurin nominal concentration	10 mg/L
3	Volume of resazurin to the test wells	20µL
4	Resazurin incubation period with test plates	40 minutes
5	Positive control	Copper sulphate
6	Negative control	Sterile water

3.5.1.3 Exposure of yeast *S. cerevisiae* to Kilombero samples

The proposed optimized parameters for yeast bioassay were used to assess potential impact of environmental samples on metabolic activities of *S. cerevisiae*. Dry season sediment (n=20), soil (n=17) and water (n=27) samples from Kilombero valley were tested on the newly developed yeast bioassay. Such samples were selected because they showed elevated toxicity responses in the standardized bioassays compared to rainy season samples as presented in Section 3.2.1. Exponentially growing *S. cerevisiae* was exposed for 4 hours to water and sample elutriates (soil, sediments). Inhibition of metabolic activity was calculated in terms of resorufin fluorescence inhibition compared to negative controls.

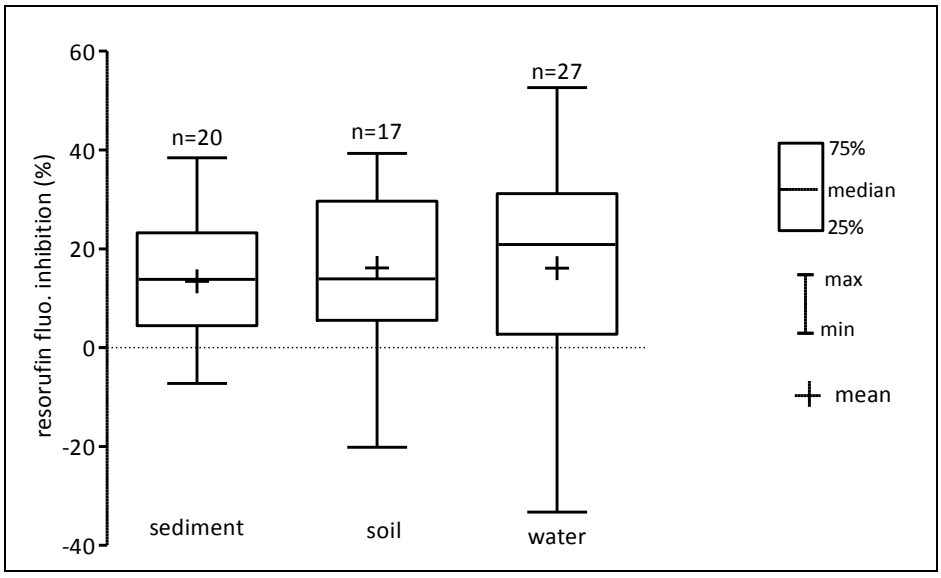


Figure 3.32: Box-whisker plots showing percentage resorufin inhibition upon exposure of yeast, S. cerevisiae to dry season Kilombero valley samples (n=64)

Dry season, sediment, soil and water samples, exhibited 13.41%, 16.12% and 16.09% mean inhibition of yeast, *S. cerevisiae* metabolic activity, respectively. The observed maximum resorufin

fluorescence inhibition by sediment, soil and water samples were 38.44%, 39.32% and 52.65% respectively. However, 3 out of 20, 2 out of 17 and 6 out of 27 sediment, soil and water samples, exhibited 5%, 10% and 18% mean stimulation of yeast metabolic activities, respectively, while the rest of sediment, soil and water samples showed 17%, 20% and 26% mean inhibition, respectively (Fig. 3.32). Although water samples exhibited an elevated inhibition of yeast metabolic activities, there were no statistically significant differences between the mean inhibition shown by sediment, water and soils *(p>0.05, 1-way ANOVA, post-test-Dunn´s multiple comparison test).* Additionally, there were no significant differences between the mean fluorescence inhibition exhibited by diluted and undiluted sediment, soil and water samples (p>0.05, 1-way ANOVA, post-test-Dunn´s multiple comparison test).

3.6 Cluster Analysis

The objectives of this analysis were: (i) to evaluate the interrelationships between results of biological and analytical parameters of 45 samples (sediment and soil) from Kilombero valley, and (ii) to establish the interrelationship between sampling stations and among samples.

Hierarchical cluster analysis (n=45 samples) revealed close relationship between electrical conductivity, grain size (<20 µm) and the responses shown by *A. globiformis, V. fischeri* and *S. cerevisiae* bioassays, but not with *P. subcapitata* (Fig. 3.33). The responses of *P. subcapitata* bioassay scatter at a far dissimilar Euclidean distance of 78.08 from other parameters. C/N ratio, salinity and pH of the samples cluster together are the reason that might have influenced the outcome of the bioassays.

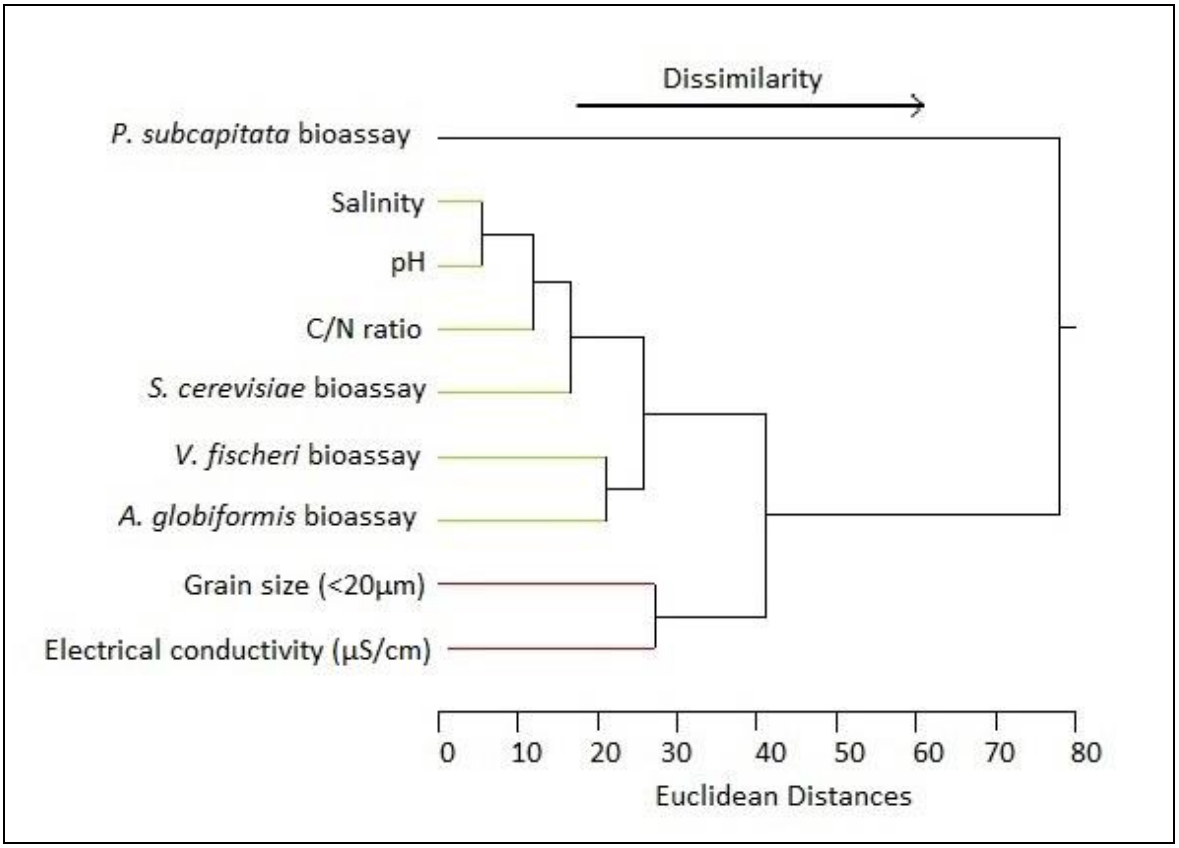


Figure 3.33: Dendrogram showing relationship between bioassays responses and measured physical parameters of 45 sediment and soil samples (Linkage: average)

Analytical and bioassay results of sediment and soil samples were included in the second part of this analysis. Since not all biological and analytical parameters were similar for water, sediment and soil samples, then water samples were excluded in order to reduce skewedness of the cluster sorting that could arise from rows with missing values. All parameters tested were applied to both sediment and soil samples. Parameters involved in the cluster analysis were: bioassays results (*P. subcapitata, A. globiformis, V. fischeri* and *S. cerevisiae*) and analytical assessment results (grain size <20 µm, C/N ratio, salinity (%), pH and electrical conductivity (µS/cm). Figure 3.34 shows the members of different clusters. If the dendrogram is cut at Euclidean distance of 25, it gives rise to four main clusters as listed below:

Cluster 1: Incudes sample KSC9 which separates itself from the rest of the clusters. This sample joins 42 other members of the cluster at a Euclidean distance of 45.1. This cluster is governed by moderate inhibition of *V. fischeri* but elevated toxic response to *A. globiformis* and *P. subcapitata*

bioassays, highest C/N ratio of 22.10 in relation to other members. This is a dry season sediment sample, which was assigned to class 3: elevated critical risk according to FRB- classification system.

Cluster 2: Consists of 16 members of the cluster, namely samples IP1, KSC2, KSC10, KPL6, KPL1, IP4, KSC1, KPL8, KSC1, KPL8, KSC5, KSC4, KSC7 and IP3, which join at a distance of 23.67 to the closely related samples MBS1 and IP2. This cluster is characterized by low to moderate inhibition responses in *V. fischeri, A. globiformis* and *S. cerevisiae* bioassays, $\leq$ 10% stimulation of *P. subcapitata* bioassays, > 50% of particle size distribution in these samples have <20 µm diameters, and electrical conductivity ≥ 40 µS/cm. Additionally, it is dominated by dry season samples.

Cluster 3: Consists of 26 members. Closely related samples KPL2, KP2, TAC2, TAC4, KPL7, TAC1, KPL3, IP5, TAC3, MBS6, KPL9, IP7, KP1 and KSC8 join other eleven samples at Euclidean distance of 17.9. These closely related samples are KSC3, KP4, MBS7, KSC6, KPL5, KPL10, MBS5, KP3, MBS3, MBS4 and IP6, which are clustered together. The members of this cluster are characterized by >50% and >10% stimulation of *P. subcapitata* and *S. cerevisiae* bioassays, respectively, slightly more acidic (pH <5) compared with other samples and have C/N ratios between 15 and 17. It is dominated by rain season samples.

Cluster 4: Consists of MBS9, sample, which separates itself at Euclidean distance of 25.1 from the rest of the members of cluster 3. This is a dry season soil sample which showed a highest stimulation (>100%) of *P. subcapitata* and a low toxic response (3% inhibition) to *A. globiformis* bioassay when compared to the rest of the samples. Additionally, this sample has a relatively wide C/N ratio (17.84) in comparison to the members of cluster 3.

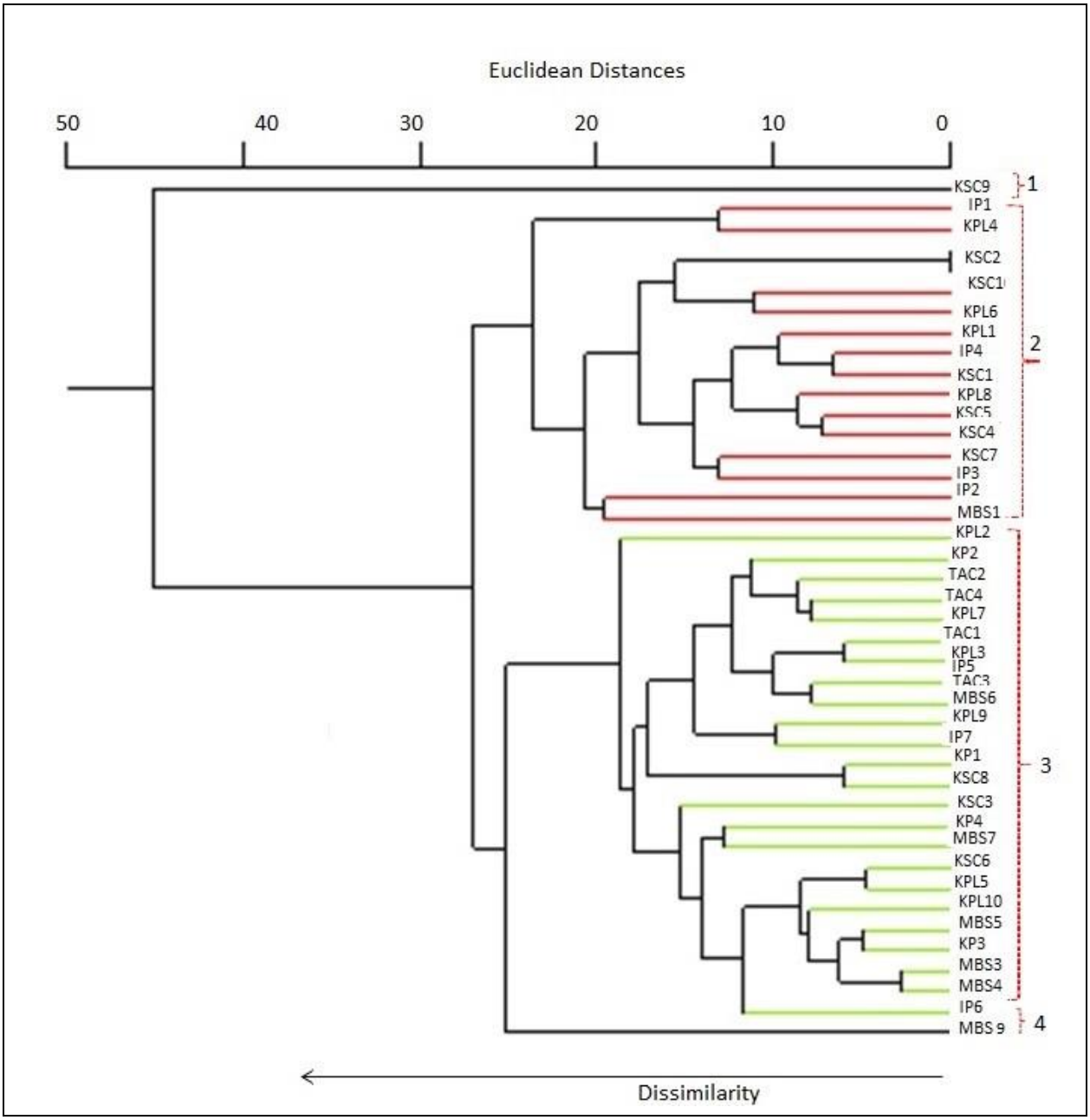


Figure 3.34: Dendrogram showing the clusters of 45 samples (sediments and soils) from different sampling stations according to bioassays results (P. subcapitata, A. globiformis, V. fischeri and S. cerevisiae) and analytical parameters results (grain size <20 µm, C/N ratio, salinity (%), pH and electrical conductivity (µS/cm). (linkages: average)

3.7 PCA of bioassays and analytical sediment data for Kilombero samples

The results of four bioassays, AGI, LBT, BCA and Yeast tests for sediment and soil samples were included in the PCA. Bioassays results from water samples were excluded because there were no results for metal screening. Moreover, elements such as sulphur, nickel, and molybdenum, which were detected only in few samples, were also excluded from PCA, in order to reduce the likelihood of skewedness of the PCA results. The 16 screened elements (metals and metalloids) and their detection frequencies have already been shown previously in Fig 3.34. Individual sediment and soil samples with at least a minimum of 17 out of 22 variables were used for PCA. Components were extracted by matrix of correlation. Scree plots of the total variance explained by each factor, and the proportion of total variance accounted by each factor, were used to deduce factor components. Moreover, PCA revealed 6 components with eigenvalues greater than 1 and three factor components that explained a total variance of 28.7, 23.0 and 13.1, respectively. A maximum of three factors were determined by the weight of component loadings greater than 0.5. A scree plot that indicates the eigenvalues of the factor loadings is presented in Fig. 3.35.

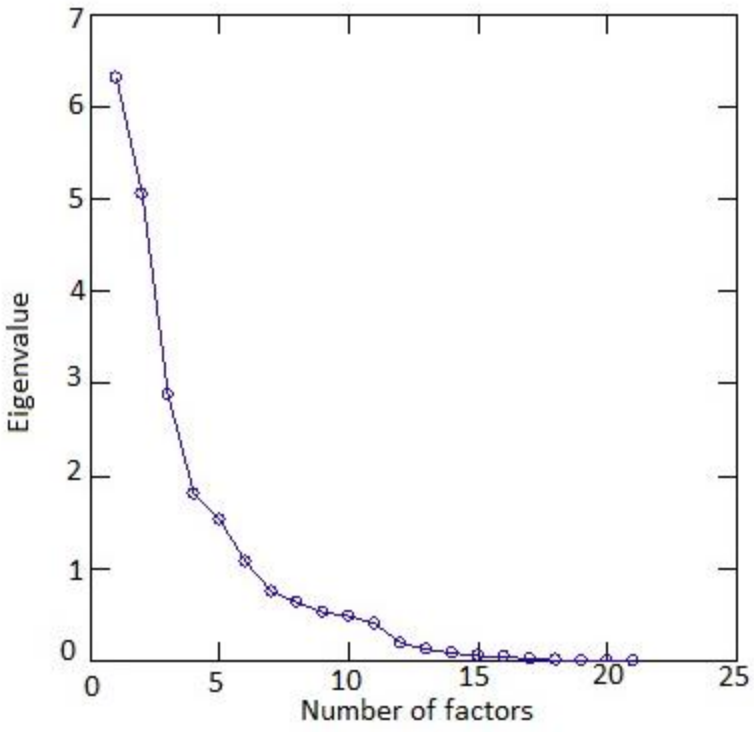


Figure 3.35: Scree plot for bioassays and analytical data in PCA for Kilombero samples

Orthogonal rotation of the components did not provide any easy interpretation, therefore the component loadings were maintained without rotation. The three component loadings are presented in Table 3.26, and can be summarized as:

- Component 1: Included grain size <20 µm, which corresponded with BCA (*A. globiformis* bioassay), heavy metals such as Co, Cu, Fe, Zn and other earthly elements such as K, Sr, Th, Ti and Zr
- Component 2: Had C/N ratio, *V. fischeri* bioassays (LBT) and other metals such as Ca, Co, Fe, K, Cr, Mn, Rb and V
- Component 3: Consisted of *P. subcapitata* bioassay (AGI), which was negatively associated with Pb and V

Yeast test, *S. cerevisiae* bioassays did not associate with any of the parameters in the PCA.

Table 3.26: Component loadings of each PCA parameter

	Component loadings (un-rotated)		
Parameter	Component 1	Component 2	Component 3
% Grain size <20 μm	**0.870**	-0.184	0.016
C/N ratio	-0.267	**0.645**	0.177
AGI	0.074	0.152	**-0.798**
BCA	**0.552**	0.158	-0.484
LBT	-0.429	**0.635**	0.378
Yeast test	-0.218	0.375	0.047
As	0.375	-0.090	0.321
Ca	-0.277	**0.686**	-0.376
Co	**0.743**	**0.550**	-0.039
Cr	0.268	**0.520**	-0.436
Cu	**0.502**	0.309	-0.056
Fe	**0.667**	**0.682**	-0.023
K	**-0.631**	**0.693**	0.130
Mn	0.427	**0.617**	-0.156
Pb	0.178	-0.361	**0.675**
Rb	-0.101	**0.759**	0.422
Sr	**-0.910**	0.318	0.171
Th	**0.583**	-0.473	0,421
Ti	**0.768**	-0.046	0,090
V	0.287	**0.543**	**0.666**
Zn	**0.585**	0.395	0.215
Zr	**-0.775**	-0.235	-0.120

Bold =major loadings (above 0.5)

A two-factor analysis was used to find out which stations or samples clustered together according to parameters listed in Table 3.26. Among sediment and soil samples (n=42), KSC9, IP3, MBS1 were not closely related to the rest of the samples which cluster together in factors (2). KPL4 and KSC4 were closely related, while IP2, MBS7 and KPL6 clustered together (Fig. 3.36). The characteristics, which govern this orientation, were that: MBS1 had highest concentration of Rb and had shown relatively high inhibition response in *V. fischeri* bioassays. IP2 and MBS7 had highest concentration of Fe and K respectively and both showed moderately higher inhibition of *V. fischeri* bioassay, than other samples. Samples KPL4 and KSC4 both had elevated concentration of Ti, while IP3 had the highest concentration of Mn as opposed to other samples. KSC9 separated itself from the rest of the samples, although it had the same range of metal concentrations as other samples, because of its widest C/N ratio and high inhibition responses shown in *V. fischeri, A. globiformis* and *P. subcapitata* bioassays.

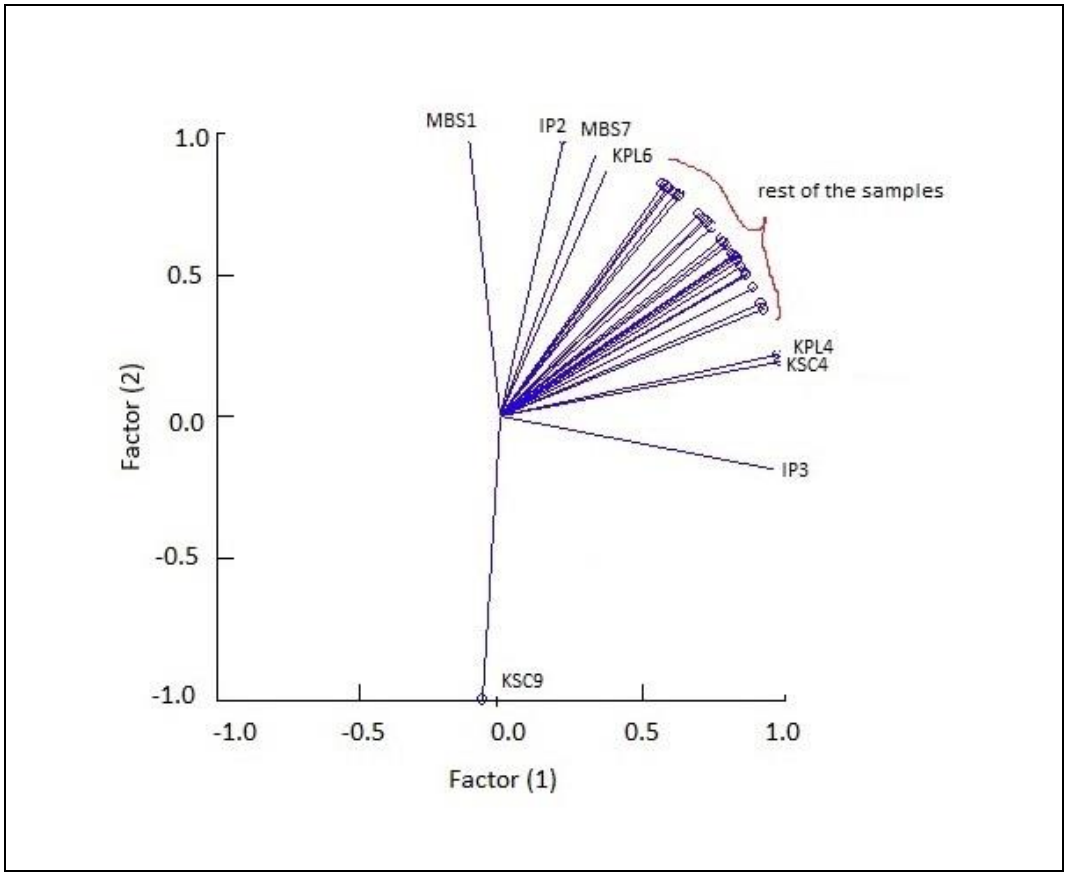


Figure 3.36: Two factor loading plot (rotated-varimax) for soil and sediment samples (n=42) according to metal/elemental screening, C/N ratio, grain size (<20 µm) and bioassays results.

3.8 Field surveys and questionnaire analysis

3.8.1 Land cover and large scale agricultural practices

Table 3.27: Overview of the estimated land cover/use of the Kilombero Valley Ramsar Site, Tanzania

Land use/cover	Area (ha)	% Proportion
Urban areas	100	0.01
Water: rivers/streams	1,680	0.16
Permanent swamps	4,160	0.40
inundated wood land	7,570	0.72
Bush land	53,110	5.07
Cultivated land	72,740	6.95
Forest	84,300	8.05
inundated bush land	149,970	14.32
Grassland	207,980	19.86
Woodland	214,000	20.43
inundated Grass land	251,670	24.03
Total	**1,047,280**	**100**

Source: (URT, 2013)

About 40% of the Ramsar site-valley is flooded during rainy season, thus resulting to inundated lands (Table 3.27). Approximately 7% of the total land cover of the Ramsar site is currently used for agriculture, and only about 0.01% of the area is residential or urban areas (Fig. 3.37).

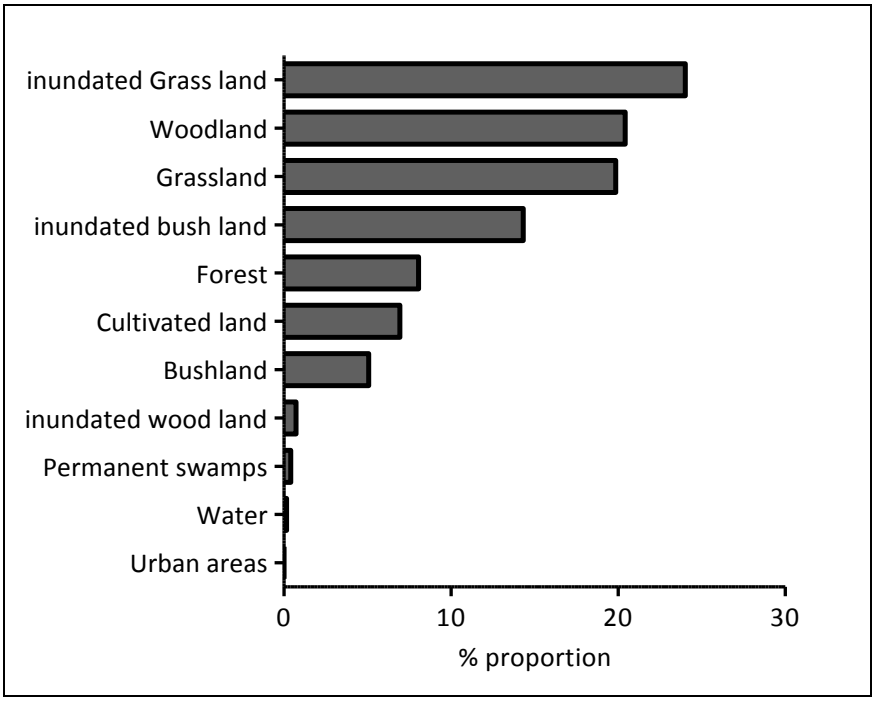


Figure 3.37: Percentage proportion of the estimated land cover or land use for Kilombero Valley Ramsar site Tanzania. (URT (2013))

Fig. 3.38 indicates that Teak Co. owns and uses largest piece of land in the valley, followed by KPL and KSC. Although, the majority of teak plantations are located in the Ulanga District, outside the Ramsar site, such plantations are close to tributaries, which drain into Kilombero River that runs about 65 km down the stream in the Ramsar area.

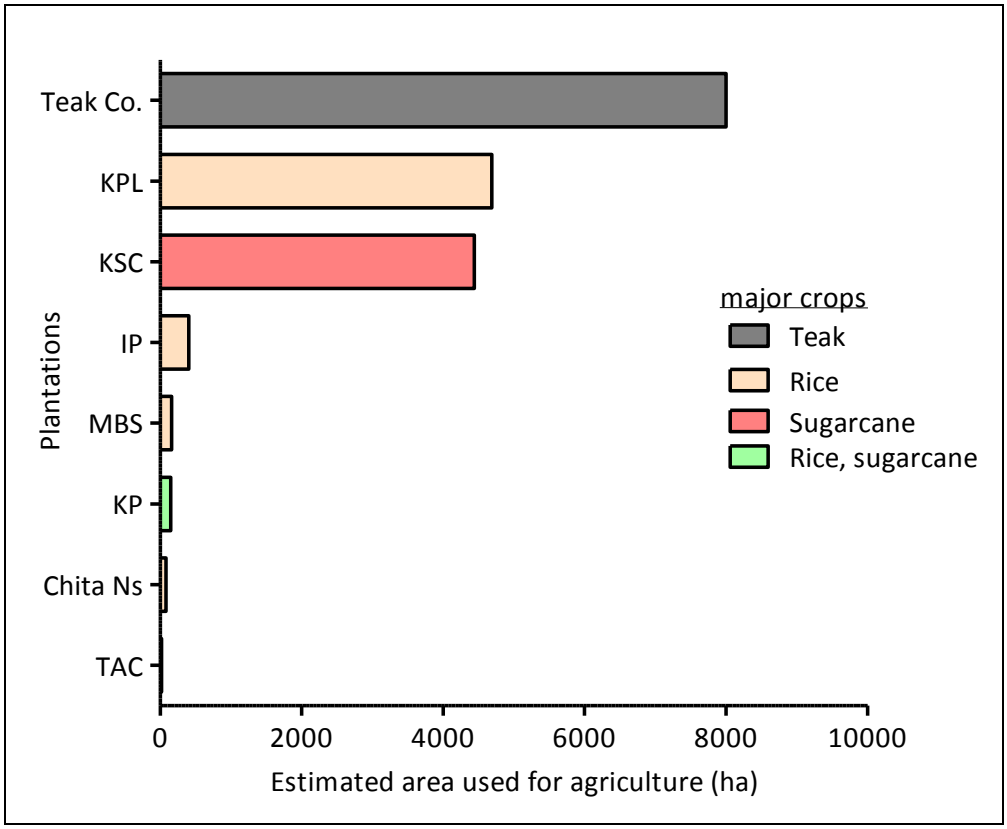


Figure 3.38: Estimated land area used for major crops cultivation in the surveyed plantations. KPL= Kilombero Plantations Ltd, Teak Co.=Kilombero valley teak Company, Chita Ns=Chita national service farms, TAC=Tanganyika Agricultural Research Centre, MBS=Mbingu sisters farms, IP=Idete agricultural prison, KP=Kiberege agricultural prison, KSC=Kilombero sugarcane plantations

3.8.2 Frequently used agrochemicals by the surveyed plantations

Following the interviews with the company agronomists /agricultural officers, and information from the questionnaires, the following pesticides shown in Table 3.28 were listed as the most frequently used brands by the study plantations. Details of individual plantation on type and extent of pesticides used will not be listed in this study, because it was promised that such information would be treated anonymously. Based on this survey, other listed agrochemicals and their active ingredients in the brackets are summarized in Table 3.28.

Table 3.28: List of frequently used pesticides (trade names) in the surveyed plantations

Herbicides:	• 2, 4-D (2, 4-dichloro phenoxyacetic acid) • MCPA 750 used as Dimethylamine salt (2-methyl-4-chlorophenoxyacetic acid) • Parapaz (paraquat) • Ronstar 380 FLO (oxadiazon) • Roundup ® or Touchdown® (glyphosate) • Sencor 480SC (Metribuzin) • Servian 75 WG (halosulfuron) • Sprayfilm 10 (Terpenic polymer) • Triachlor–M (metolachlor) • Triclopyr 480 (triclopyr acid, triclopyr triethylamine salt) • Velpar (hexazinone + diuron) • Volmethalin / Pendimethalin (37% Pendimethalin (N-(1-ethylpropyl)-3,4-dimethyl-2, 6-dinitrobenzenamine) • Volmetra 500SC (Atrazine 235g/l + Ametryin 245g/l)
Fungicides:	• Amistar 250 SC (azoxystrobin) • Copper Oxychloride • Copper Sulphate • Prostar® 70WP (Flutolanil 70%)
Insecticides:	• Actellic Liquid 50EC (Pirimiphos-methyl) • Dursban-4E (chlorpyrifos) • Gamal M 20 • Gaucho FS-350 (imidacloprid) • Jumbo 480 (clomazone) • Simuthion (Fenitrothion)

Underlined: detected pesticides in the collected samples, active ingredients are in brackets

The aforementioned pesticides were revealed during interviews, but other pesticides that are used in the plantations might not have been declared. More information on pesticide registered for use in rice and sugarcane farms are shown in Appendix 6.

3.8.3 Means of agrochemicals application

Aerial application of pesticides and ground spraying boom were revealed to be used in KSC and KPL. Trigger pump/compressed air was listed to be used by Teak Co., while knapsack spraying and spot application were used by all surveyed plantations. Sometimes pesticides brands were mixed before spraying, and this was revealed during face-to-face interviews on which 90% of the surveyed

plantations were very candid. Reasons for pesticide mixing as responded in the questionnaire survey were:

- to increase the efficacy of expected outcome, depending on the severity of the pest/weed problem the pesticides were mixed;
- due to uncertainty about the quality of a certain pesticides for a particular pest; and
- due to presence of counterfeit or forged products in the market that did not function as recommended in the leaflets or instruction label.

Sometimes pesticide brands were changed, following recommendation from agricultural officials or when it happened that the old brands were no longer effective to a target pest.

Moreover, during field survey it was revealed that different types of fertilizers were applied in the plantations, and that the choice of fertilizer depended on soil-type or low soil productivity that has resulted in decreased crop yield in the preceding crop season/cycle. Most listed brands of fertilizers were:

- NPK
- Urea
- Diammonium Phosphate (DAP)
- Muriate of Potash- potassium chloride (MOP)
- Aqua Matrix
- TAC-*Ammonium Chloride*
- *Minjingu* phosphate fertilizers

These fertilizers were applied in the farms by either aerial /foliar spray, localized placement, sprinkling or broadcasting depending on the proportion of the plot. Soil type and the expected yield, had a high influence on type and amount of fertilizer to be applied in an area. Surface run-off during high precipitation can dislocate such pesticides and fertilizers downstream. Table 3.29 summarizes an overview of some of nutrient detected in the steams adjacent to agricultural field.

Table 3.29: Concentrations of detected nutrient levels in the water samples collected from Kilombero Ramsar site during dry season sampling. IP=Idete Agricultural prison, KPL= Kilombero Plantations Ltd, KSC=Kilombero sugarcane plantations

Station-sample	NO_2^- (mg/L)	PO_4^- (mg/L)	NO_3^- (mg/L)	NH_4^- (mg/L)
Test-kit detection range	0.5-25.0	5.0-120	3.0-90.0	0.2-7.0
KPL-w1	bdl	7.0	Bdl	1.1
KPL-w2	bdl	5.0	Bdl	0.9
KPL-w3	bdl	6.0	Bdl	Bdl
KPL-w4	bdl	5.0	Bdl	Bdl
KPL-w5	bdl	6.0	Bdl	Bdl
KPL-w6	bdl	8.0	Bdl	Bdl
KPL-w7	bdl	bdl	Bdl	0.3
IP-w1	bdl	bdl	Bdl	1.0
IP-w2	0.5	bdl	Bdl	0.3
IP-w3	bdl	bdl	Bdl	0.3
KSC-w5	bdl	bdl	3.0	Bdl
KSC-w6	bdl	bdl	Bdl	0.4
KSC-w7	0.5	bdl	4.0	Bdl
KSC-w8	bdl	bdl	4.0	Bdl

bdl= below detection limit

3.8.4 Meteorological information

Summary of collected meteorological data during sampling season, 2012 and 2013 are presented in this subsection. Figs. 3.39 to 3.42 provide an overview of meteorological data on temperature, rainfall pattern, solar radiation and intensity, soil temperature, relative humidity and wind speed from the study area during sampling seasons. These data were retrieved from daily meteorological records of two sampling stations, KPL (Kilombero Rice plantations, Mngeta farms) and KSC (Kilombero sugarcane plantations, Kidatu K1-estate farms). The rest of the sampling stations had no meteorological units. Nevertheless, KPL data (solar radiation, humidity, temperature and rainfall) could represent Chita Ns and Mbingu sisters farms, while KSC data (rainfall, humidity, temperature and wind speed) could represent KP (Kiberege agricultural prison farms). Meteorological data from these two stations might not be a good representative of TAC, Teak Co, and IP, because they are located at different terrain and have different relief features from other stations.

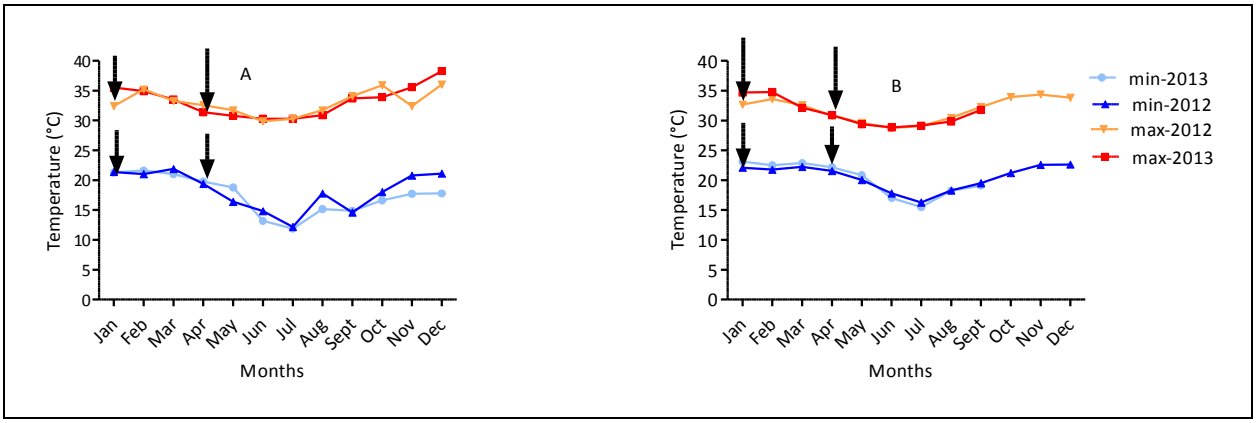


Figure 3.39: Mean, maximum and minimum temperatures (2012/2013) for KPL and KSC plantations. Data were extracted from daily meteorological records of (A) Kilombero Rice plantations (Mngeta office weather-chart record) and (B) Kilombero sugarcane plantation (K1-Estates stations). Arrows indicate conditions during sampling seasons.

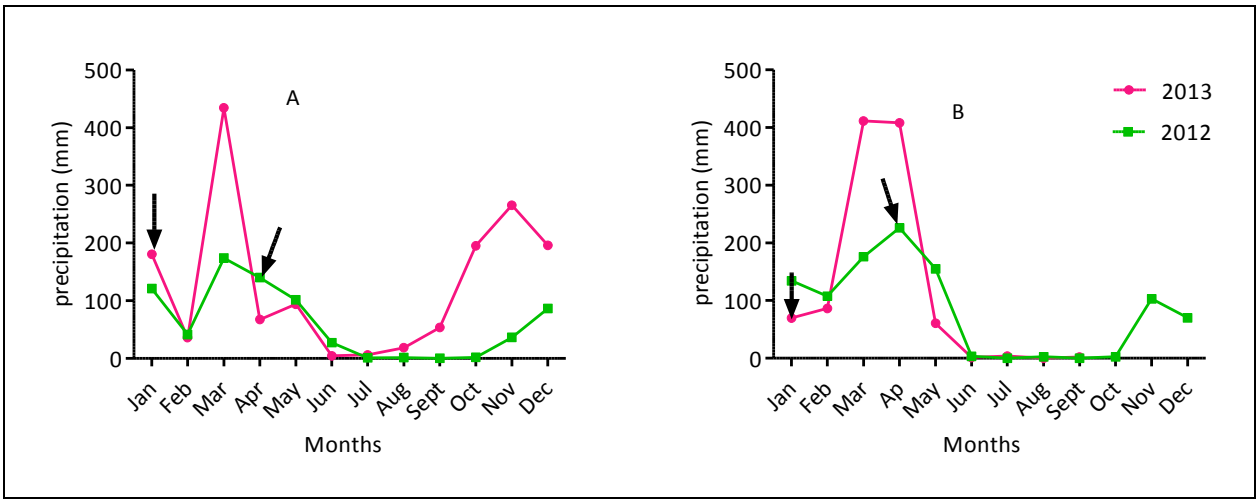


Figure 3.40: Monthly total rainfall pattern (2012/2013) for KPL and KSC plantations. Data were extracted from daily meteorological records of (A) Kilombero Rice plantations (Mngeta office weather-chart record) and (B) Kilombero sugarcane plantation (K1-Estates stations.). Arrows indicate conditions during sampling seasons.

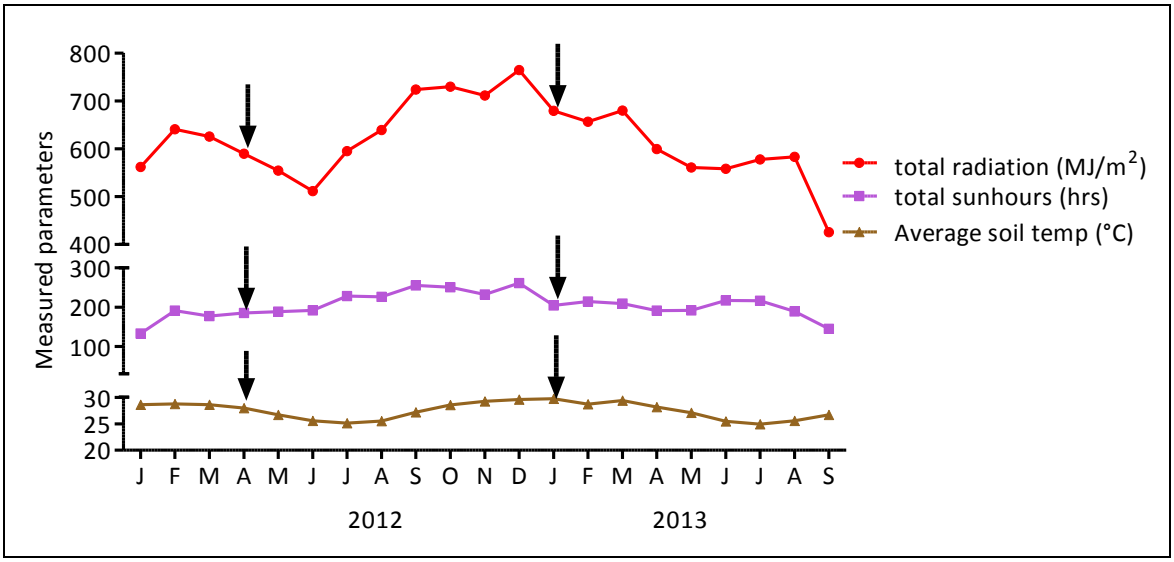


Figure 3.41: Photo radiation intensity and soil temperature parameters as recorded by Kilombero sugarcane plantation (K1-estate) for the year 2012-September 2013. Arrows indicate conditions during sampling seasons.

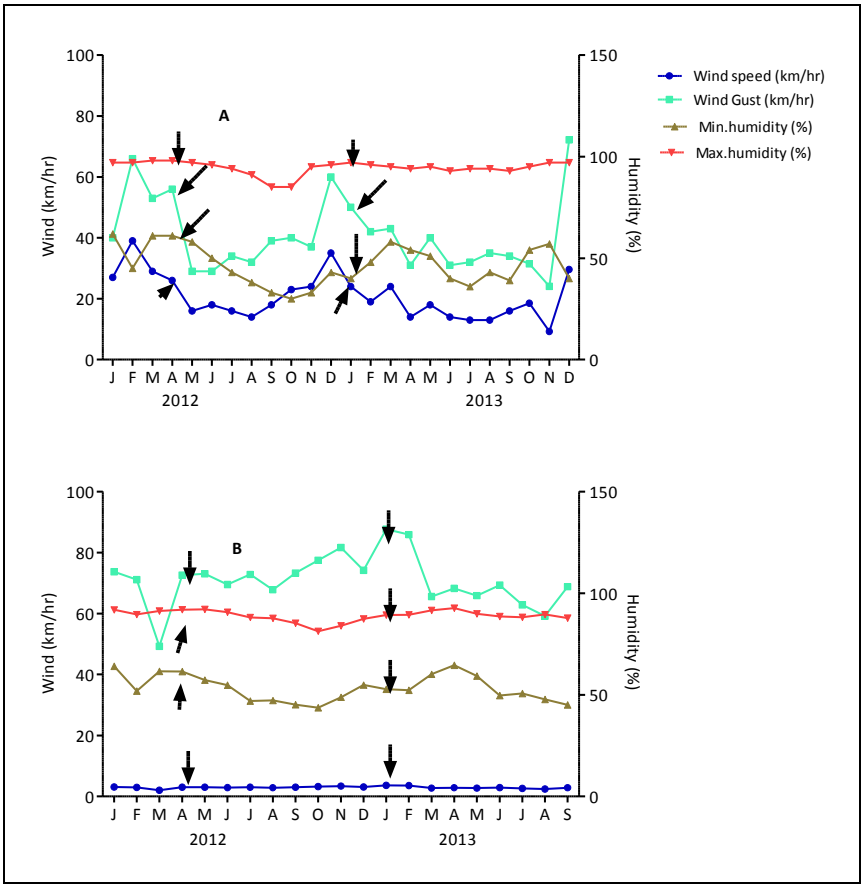


Figure 3.42: Wind and humidity parameters (2012/2013) for KPL and KSC plantations. Data were extracted from daily meteorological records of (A) Kilombero Rice plantations (Mngeta office weather- chart record) and (B) Kilombero sugarcane plantation (K1-Estates stations). Arrows indicate conditions during sampling seasons.

CHAPTER FOUR: DISCUSSION

4.1 Introduction to chapter

Despite the fast development of eco-toxicological research in the last three decades, little has been done on tropical African ecosystems. The use of bioassays for risk assessment is widely accepted by regulatory authorities of developed countries, but its application in developing countries is still lagging behind for some countries, and absolutely novel to Tanzanian setting. Eco-toxicological study of soil, sediment and water samples from tropical freshwater wetland ecosystems, where both large scale and small scale rice and sugarcane farming is ongoing, was conducted. The following specific objectives were addressed:

(i) Assessment of eco-toxicity of water, soil and sediment samples from plantations applying agrochemicals (ii) evaluation of the suitability of temperate biotest batteries for assessing pesticide contamination in tropical agronomic systems, and (iii) estimation of potential risks to humans and aquatic life based on chemical analysis data of soil-sediment-water samples from agricultural fields. In order to fulfil these objectives, samples were collected from KVRS, in Tanzania. All eco-toxicological and analytical assessments were conducted in Germany.

In this chapter, toxicity responses of bioassays are discussed based on sampling seasons and sample matrices. The overview outcome of the classification of the toxicity responses using fuzzy rule-based model is summarized on the map of sampling stations. Patterns of observed trends of bioassays results are critically evaluated. Human and environmental potential risks of agricultural activities in the Ramsar wetlands are discussed based on the results of pesticides and trace metals/metalloids screening. Lastly, the current Tanzanian government agricultural policies and pesticide management are analyzed in terms of land use and sustainability of Ramsar wetland agriculture.

4.2 Eco-toxicity of Kilombero samples

4.2.1 Bioassays assessments

As shown in the results (Figs. 3.5 and 3.6), *V. fischeri* and *P. subcapitata* showed moderately equal responses to both dry and rainy season samples (water and sediment). Dry season sediment samples (Fig. 3.5) showed higher mean inhibition responses to *A. globiformis* than rainy season samples. *P. subcapitata* was stimulated by both rainy and dry season samples (soil, water,

sediment). A few sediment and water samples showed extreme or elevated toxic or stimulatory responses to the three bioassays (Fig. 3.4). *P. subcapitata* was stimulated by all soil samples while *V. fischeri* and *A. globiformis* bioassays were inhibited by the same samples (Fig. 3.7). The toxic responses shown by the three bioassays differed significantly depending on the sample matrices and season. The possible reasons of the observed higher toxicities by the dry season samples than the rainy season samples are discussed. The samples, which showed elevated toxicities, are identified, and a detailed discussion, especially on the possible causes of such high toxic responses, follows.

The toxicity responses of *A. globiformis* indicate the presence of inhibiting contaminants in the sediments. *A. globiformis* is sensitive to both organic and inorganic contaminants (Marques *et al*., 2014; Neumann-Hensel & Melbye, 2006; Ronnpagel *et al*., 1995). Likely candidates that may be responsible for this effect in the area are non-selective, broad spectrum herbicides such as glyphosate, which are applied to the fields in the dry season before seedling. As to many agronomic systems of Tanzania, seedling season starts on the fall of first or second rains and thus for Kilombero valley is from end of February to end of March. Glyphosate and its metabolic product AMPA have strong soil adsorptive characteristics due to its large Koc, up to 60,000 L/kg (Giesy *et al*. (2000). It is likely that dry season sediment and soil samples collected after application of broad spectrum herbicides, (e.g. glyphosate), before the lapse of their degradation half-life in soils, (44 days for glyphosate (Kollman and Segawa, 1995), would have resulted in high inhibition to *A. globiformis* bioassays. As responses in the questionnaires indicate, glyphosate was applied into rice fields in a period of several days (20 to 30 days) before planting or was applied just before the onset of first rains or land ploughing for seedling. The loss of effective substances from soil when heavy rainfall floods the area, followed by dilution within the aquatic system, can explain the reason for lower toxicities during rainy than dry season. Increased leaching, drain-flow and surface run-off have been identified as the major pathways leading to e.g. herbicide transport (Carter, 2000; Gouy *et al*., 1999). How much each of these processes contributes to the run-off depends on the soil properties, the physico-chemical characteristics of the substances and on the formulation (Gouy *et al*. 1999). Carter (2000) claims that the loss of herbicide from soil is typically less than 0.1 to 1%, but can occasionally go beyond 5% of the applied mass. After emission to the water, eroded particles and dissolved contaminants are transported along the river channels during rainy season, while dilution within the residual or stagnant water during dry conditions is much lower, thus increasing

the localized impact. Therefore, it is likely that sediments collected during rainy season have fewer or lower concentrations of bioavailable organic contaminants than dry season samples. Weber *et al*. (1965) pointed out that high humus content and temperatures increase the ability of organic compounds, such as pesticides, to be adsorbed by soil particles through ion exchange forces and the equilibrium is influenced by temperature and exposure time.

Additionally, the dry season water and sediments samples that showed extreme or elevated toxic responses in the three bioassays were identified as those collected either from the outlets streams that receive drains from the rice paddies, or adjacent to pesticide mixing /sprayer filling point or from the small forests located within the KPL rice farms. Both rainy and dry season sediment samples collected in the banks of the rivers that flow within the KPL and KSC agricultural fields, showed elevated toxicity in *A. globiformis* bioassay. This indicates that contaminants are transported from their points of origin downstream and are potentially deposited in the river banks, especially when the discharge rate is low or flow velocity decreases during dry seasons.

Elevated toxicity responses shown by dry season water and sediment samples to the three bioassays might also be contributed by the presence of mixture of different types of pesticides residuals in the samples matrices, as revealed by pesticides screening results. For instance, highest inhibition (74%) of *P. subcapitata* was observed in one sediment sample from Kilombero sugarcane plantations (KSC9) where a mixture of atrazine (0.46 µg/g DW) and glyphosate (0.19 µg/g DW) were detected. As shown in Table 3.15, sediment samples from Idete prison rice farms (IP3, IP2, IP9, IP11), Kiberege prison rice farms (KP4), Kilombero rice plantations (KPL8, KPL9, KPL11 and KPL19), Kilombero sugarcane plantations (KSC2, KSC4, KSC5, KSC12, KSC15), where pesticides such as chlorpyrifos, glyphosate, atrazine and diuron were detected, showed moderate to elevated toxic responses to *A. globiformis* and *V. fischeri,* according to the fuzzy rule base toxicity categorization. 100% inhibition of *A. globiformis* implies that organisms were physiologically inactive upon exposure to contaminated sample matrices, with either the detected pesticides or any other undetected toxic substances. The observed stimulation of *A. globiformis* by a few rainy season sediment samples might be due to degradation of organic pollutants in the samples, or due to excess nutrient in the test matrices as described by Dubé & Culp (1997). The same stimulation responses in *A. globiformis* and *P. subcapitata* bioassays have been observed in studies using

temperate sediment samples (Ahlf & Heise, 2005; Hsu *et al*., 2007) and also in other type of laboratory bioassays (Stuijfzand *et al*., 2000).

Some researchers state that the co-occurrence, interaction and combined effects of different interaction between xenobiotic at any stage of the toxicological process could result in a critical hazard of the mixtures, increasing ecological risks (Chesworth *et al*., 2004; Gatidou & Thomaidis, 2007). Elevated toxicity response shown by one sample in one bioassay such as *V. fischeri,* is not comparable to elevated toxic responses observed in another bioassay such as *P. subcapitata* or *A. globiformis*. Different organisms show different sensitivities, that is why biotest batteries are used for testing of environmental samples. In addition, the response range differs among bioassays as has been shown by Ahlf & Heise (2005).

Cluster analysis (Fig. 3.34) was able to differentiate between rainy and dry season samples, showing that there is a difference between them. Cluster 2 was dominated by dry season samples, because they showed at least moderate toxicities in two of the bioassays, while rainy season samples dominated cluster 3, which showed stimulation in *P. subcapitata* bioassays and at least low or no toxic response in the *A. globiformis*, *V. fischeri* and *S. cerevisiae* bioassays

4.2.2 Stimulation in the algae, *P. subcapitata* bioassay

Of all rainy and dry season sediments, soil and water samples (n=143), collected from 8 sampling stations, 131 samples (sediment, soil and water) showed stimulation in the algae test, while 12 samples (sediment, soil and water) showed inhibition of *P. subcapitata* in the chronic, 72 hours exposure.

Although, algae are widely used in eco-toxicological tests, some researchers such as Ma *et al*., (2006b), argued that complete inhibition of algae by environmental samples might require a high concentration (EC_{50} =5.5 mg/L, 96 hours) of some herbicides such as glyphosate. Low doses of some toxic compounds (below the reported EC_{50} concentrations), such 2, 4-D, glyphosate, diuron in sample matrices have the ability to induce hormesis in plants, due to their plant growth regulator properties (Draber, 1983; Duke *et al*., 2006). Moreover, Malik (1989) and Marsalek (1996) showed that upon degradation, glyphosate at low doses below 5 mg/L, can act as a source of carbon or nitrogen to algae. Also the study on impacts of metribuzin, a photosystem II inhibitor, on green algae and cyanobacteria (Lürling & Roessink, 2006), has shown that presence of metribuzin (100 µg/L) in the mixture resulted in greening response due to an increase of chlorophyll *a* concentration. The inhibition of electron flow in PS II at the acceptor side might lead to an increase

of fluorescence at low concentration (100 µg/L) of herbicides such as metribuzin (Lürling & Roessink, 2006) or other toxicants (Jos *et al*., 2010; Ribeiro *et al*., 2000). These circumstances might have contributed to the observed stimulations in the chronic *P. subcapitata* bioassays by samples, in which diuron and glyphosate were detected, (refer Table 3.16). With all the observed stimulation in algae test in this study, 74% inhibition of *P. subcapitata* by one sediment sample (KSC9) collected close to the pesticide filling station and adjacent to the stream that receives drains from the sugar processing plant, was considered of importance in terms of potential risk. The reason for this inhibition might be contributed by other factors such as presence of mixture of detected pesticides (atrazine and glyphosate) or any other toxicants in the same sample and low decomposition of organic matter, due to relatively wide C/N ratio (22). Soils with narrow C/N ratio of 10-18 are characterized by rapid decomposition and mixing of organic matter, and such soils have slightly acidic to neutral pH (FAO, 2006). Soils of C/N ratio of 18-29 are developed in moderately nutrient-poor conditions, in cool moist environment and are characterized by moderate to low decomposition of organic litter (FAO, 2006). With an exception of this one sample, which had a C/N ratio of 22, the rest of sampled stations in Kilombero valley had narrow C/N ratios less than 18, which is within the range of higher decomposition of organic matter according to FAO, (2006). Fig. 4.1 indicates that C/N ratio varied from 10 to 20 for the samples that were measured. There is a slight increase in algae stimulation with C/N ratio of some samples, which indicates that other factors might have contributed to the stimulation. Soils with narrow C/N ratio of 10-15 (Table 4.1) are not well drained, and therefore they can retain a number of organic contaminants or nutrients. Vegetation cover and environmental factors such as temperature and humidity determine the rate of decomposition of the accumulated litter layer leading to enrichment of surface soils with organic nutrients in a particular site (Batjes, 2012).

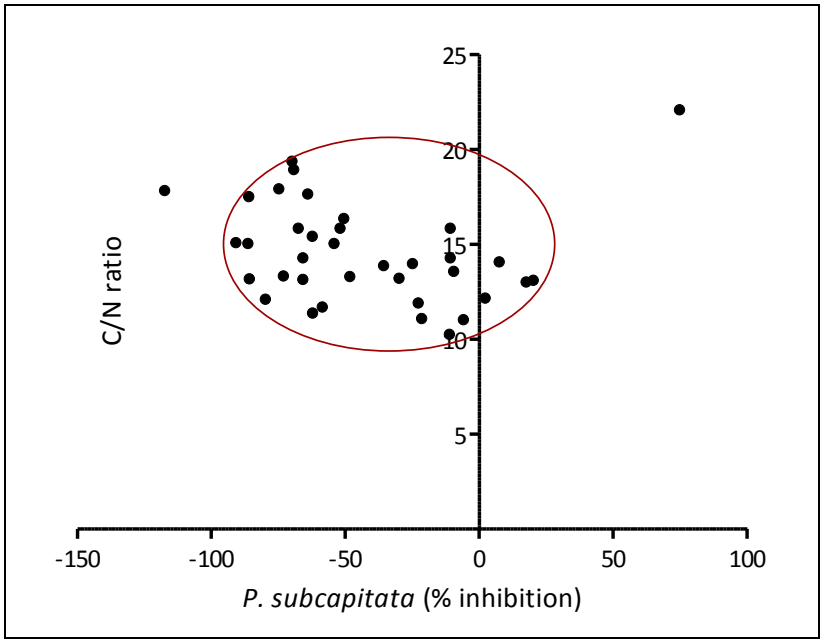


Figure 4.1: Scatter plot of P. subcapitata (% inhibition) and the C/N ratio of sediment samples

Therefore, the observed stimulation in *P. subcapitata* bioassay can be explained by presence of high amount of organic nutrients in the particular sample matrices. However, there is a great variation in the measured C/N ratios because of the influence of land-based plants or land use practices, weather patterns and soil fauna available in an area (Kamiri *et al*., 2013). For instance, excess fertilizer application increases soil salinity, which reduces microbial degradation of organic nutrients (Haynes & Naidu, 1998). Laboratory experiments with forests soils and modeling experiments on nitrogen mineralization from manures indicate C/N ratio of 20 is generally a threshold point where either net N-mineralization (if the C/N ratio is narrower) or net N-immobilization (if the C/N ratio is wider) by soil fungi and bacteria occurs (Bengtsson *et al*., 2003; Probert *et al* 2005; Raun *et al* 1998;). Different scholars demonstrated that measured C:N ratios are narrower in high clay soils and in sub soils due to microbial products such as proteins–peptides that are adsorbed by clay particles and interact with organic matter through their hydrophobic domains. (Sorensen, 1981; Stevenson, 1986; Sollins et al., 2006). The narrow C/N ratio of organic layers on surface soils may be due to the adsorption of proteins and peptides with hydrophilic and hydrophobic domains. Soils, where mono-cropping of high cellulose and lignin content crops is practiced, like sugarcane and rice plantations, are likely to have potentially wide C/N ratios above 20, compared to soils from areas where mixed farming, or crop rotation with low lignin crops like legumes, is practiced (Cambardella & Elliott, 1992).

Table 4.1: ISRIC-classification of soil according to range of physical parameters

Class	Soil properties	C/N ratio ranges	Range of electrical conductivity (dS/m)*	Soil pH (aqueous form)	% clay content range
1	Poorly drained soils	0-10	0-5	0-4.5	0-10
2	Imperfectly drained soils	10-15	5-10	4.5-5.5	10-20
3	Moderately well drained soils	15-20	10-15	5.5-6.5	20-30
4	Well drained soils	20-25	15-20	6.5-7.3	30-40
5	Somewhat excessively drained		20-25	7.3-8.5	40-50
6	Excessively drained soils	>25	<23	>8.5	>50
Estimated maximum value for agronomic activities		30	31	8.8	67

Source: Batjes, (2012), *1dS/m=1000µS/cm

However, sediments and soil samples of Kilombero valley had relatively narrow C/N ratios of 10 to 20 (Fig. 4.1), indicating nutrient enrichment during flooding or surface runoff in the rainy season, where organic humus is transported from the highlands to these lowland flood plain wetlands, thus elevating its organic nutrients. Kilombero Valley Flood plains contain soils of alluvial origin that vary in texture, from sands to clays, which are mainly influenced by the accumulation of parent materials and organic matter. According to Veldkamp, (2001) study on soil mapping of Tanzanian agro-ecological zones, Kilombero valley has diverse soil types, which can be classified as well or moderately well drained fluvisols, imperfectly drained hardpan soils and poorly drained fluvisols. Appendix 7 provides more details of major geological soil types for Kilombero Valley.

Additionally, the narrow C/N ratio of Kilombero sediment and soil samples is also enhanced by warm-humid environments with high temperatures above 28°C, which accelerate microbial degradation of organic litter. Environmental factors like humidity, temperature, soil texture, clay content, precipitation and soil-water content, have great influence on the biological degradation of organic matter (Burke *et al*., 1989).

Elevated nutrients experiments

As shown in Section 3.4.3.3, increased concentrations of dissolved organic carbon (as peptone) in the test medium up to 400 mg/L or 0.04% carbon resulted in stimulation of *P. subcapitata.* This amount was 10-fold lower than the minimum percentage of total carbon in the sediment (0.5%) and soil (1.30%) samples of Kilombero valley (Fig. 3.1). Besides, in this experiment, 24% to 47%, stimulation was observed when *P. subcapitata* was exposed to the test medium, which contain a mixture of dissolved organic carbon (peptone) and vitamins B_1 & B_{12} (Fig. 3.28). Most of B vitamins

have been shown to occur in arable soils, due to release from vitamin-containing plants and animal manures/residues, liberation from the roots of growing plants and synthesis by soil microorganisms (Lochhead & Burton, 1956; Mozafar, 1994). Although, B-vitamins were not tested in the Kilombero samples, this laboratory experiment and the reported studies on bio-stimulants i.e. materials that enhance plants growth in low concentrations such as humic/fulvic acids, cytokinins, marine algae extracts, polymers of lactic acids, amino acids, B-vitamins and ascorbic acids (Heckman, 1993; Kinnersley *et al*., 1990; Oertli, 1987; Russo & Berlyn, 1991), could be another reason for observed stimulation in *P. subcapitata* bioassay. However, the mechanism of this stimulation could not be elucidated in this study. Further experiments to test the impact of different mixtures of organic nutrient to *P. subcapitata* or to screen for B-vitamins in samples that show high stimulation to algae might help to add further information on the cause for stimulation.

Moreover, in three different laboratory experimental settings, with enriched nutrients concentrations in the test DIN medium, addition of PO_4^{2-} concentrations as low as 8 mg/L, concentration of trace elements (Fe^{2+}) and microelements by factors of 10 and 1.25 higher than the recommended algae growth DIN medium, respectively, resulted in 5% to 12% stimulation (Fig. 3.21, and 3.25). This stimulation is not significant and is much lower when compared to high stimulation up to 100% shown by sediment and soil samples of Kilombero valley. Nevertheless, it suggests that presence of low concentrations of micronutrients or trace elements, such as Fe^{2+}, Mo^{6+}, Co^{2+} and Zn^{2+} (as shown in DIN STD 2 and 3), in the soils and sediment samples matrices, might enhance algae stimulation in the chronic test. Laboratory work by Aruoja *et al*. (2004), showed that soil suspension that contained as high as 12.5 mg Zn/L stimulated the growth of algae up to 8-fold than the OECD medium. In another laboratory experiment by Hund (1997) with soil sample extracts which contain higher concentrations of macro and micro nutrients than the recommended OECD algae medium, the same stimulatory trend was observed. Therefore, for chronic algae test, nutrient concentrations may be a reason of underestimation of toxicity response. In this laboratory experiment with DIN medium STD 1 (excess micronutrients such as Ca^{2+}, K^{+}, Mg^{2+}, and Cl^{-}), 33% to 95% inhibition of *P. subcapitata* growth rate in all concentrations enriched by factor higher than 2.5. Elevated concentrations of phosphate and NH_4^{-}-N-sources resulted into inhibitions suggesting that the conditions were not favorable for algae to grow. Although excess growth of algae in aquatic ecosystems is an indicator for increased concentrations of nitrogen and phosphates (Carpenter *et al*., 1998), a study by Rabalais, (2002) has demonstrated both high and limited

concentrations of inorganic nutrients can also account for limited growth of algae in aquatic systems.

4.2.3 Fuzzy rule base classification of bioassay results

A fuzzy logic expert system is used as a tool to handle uncertain or imprecise information by allowing a subject instead of a strict decision such as yes/no, high/low, to belong to a specific category (fuzzy set) with a gradual membership value between zero and one (Keiter *et al*., 2009; Zadeh, 1965). In fuzzy rule-base systems, a variable belongs to a fuzzy set and the knowledge or information is represented by "*if*….and *then*…" rules which helps to model the uncertainties of various parameters (Adriaenssens *et al*., 2004; Ahlf & Heise, 2005; Klir & Yuan, 1995). A Fuzzy Rule Based (FRB) classification system was set up to provide logical and transparent means of interpreting the results from the array of bioassays. A very limited number of samples showed elevated toxic responses in the three bioassays. Results of a single toxicity test system might not provide enough information for risk assessment of environmental samples because of uncertainties in the test systems and in the natural environments (Adriaenssens *et al*., 2004; Tran *et al*., 2002). Hence, classification of the bioassay end point responses was of great importance in order to integrate the results of the three bioassays into the overall ecological risk posed by the Kilombero samples.

Three fuzzy toxicity categories were deduced based on the distribution of data, and on the assumption that the database consisted of a wide range of samples that showed low to high or elevated toxic responses by the test organisms. 50% of samples around the mean value (between the lower and upper percentile) were considered to represent moderate toxicity while the lower 25 and upper 25 percentiles were assumed to be equivalent to low /non-toxic and strong toxic levels, respectively (Ahlf & Heise, 2005). This assignment to different categories was based on ecotox data, collected by the Applied Aquatic Toxicology Research group (HAW-Hamburg), over a wide contamination range of river sediments, mainly from the Elbe river, comprising results from biotests with *V. fischeri (n=456), P. subcapitata* (n=230), and *A. globiformis (n=205)* (Fig. 4.2). Kilombero samples showed lower mean inhibition responses in the *P. subcapitata* and *A. globiformis* than the whole database data, while *V. fischeri* showed an overlap in the mean toxicities. These differences in toxicity responses could be due to presence of less effective (toxic) compounds in the Kilombero samples. The absolute toxicities gained when measuring same samples or samples collected from same area at different times, may be different, as the methods

may deviate a bit between different laboratories, such as those reported by Ahlf & Heise (2005), from Technical University of Hamburg-Harburg. The ranges, however, should reflect the different sensitivities in the bioassays and a categorization like this could therefore be transferable. The deduced categories with their fuzzy overlaps for Kilombero samples can be described as:

- Category 1: little or low toxic responses. Corresponded to <15±5% inhibition of bioluminescence of *V. fischeri,* <35±5% inhibition of *P. subcapitata* growth rate and <17±12.5% inhibition of *A. globiformis* dehydrogenase activity.
- Category 2: Moderate toxic responses. Corresponded to inhibition of *V. fischeri* bioluminescence of from 15±5% to 30 ±5%, inhibition of *P. subcapitata* growth rate from 35±5% to 70±5% and inhibition of *A. globiformis* dehydrogenase activity from 17% to 60 ±12.5%.
- Category 3: High toxic responses. Corresponded to >30±5% inhibition of *V. fischeri* bioassay, >70±5% inhibition of *P. subcapitata bioassay and >*60±12.5% inhibition of *A. globiformis* bioassay.

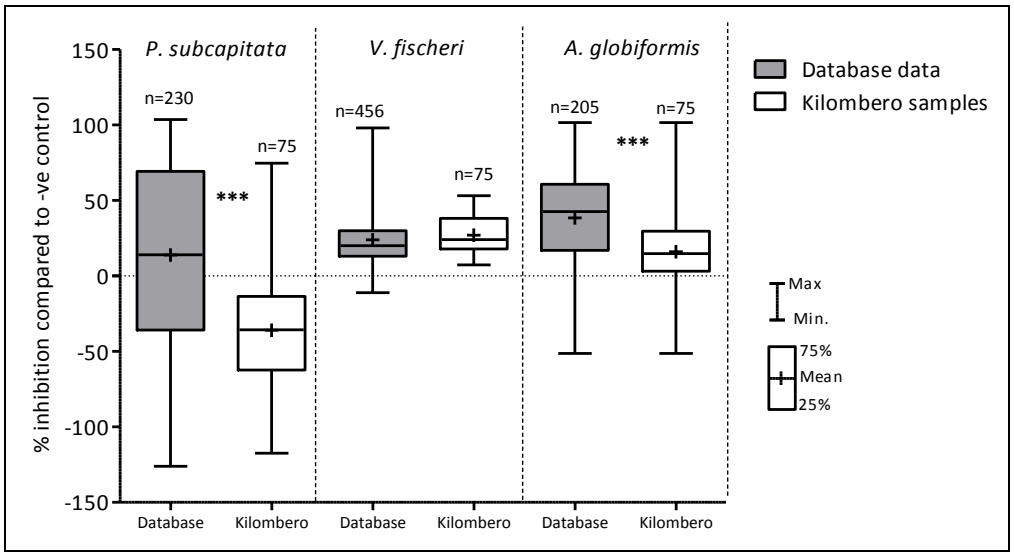


*Figure 4.2: Comparison of bioassays responses between database samples and the Kilombero valley samples (soils and sediments), ***:* p*<0.001, 1-way ANOVA, with post-test Dunn´s multiple comparison test*

An overview of the distribution of toxic classes is shown in the map of sampling stations Fig. 4.3. Under the three fuzzy logic toxicity categories of Kilombero valley samples (sediments and soils), fuzzy rules resulted in three toxic classes as described below:

- Class 1: Little or no potential risk. Consisted of 73% of results obtained from Kilombero valley samples (sediments and soils). In this class, no bioassays showed any toxic response, or at least two bioassays showed low toxic responses. The responses of the three bioassays were in the range of negative controls and replicates of the tests systems, which were mostly, restricted in the validity criteria of the biotests.
- Class 2: Critical risk. Consisted of 25% of results obtained from Kilombero samples (sediments and soils). The bioassays results in this rank included samples, which showed moderate toxic responses in at least two tests, and no bioassay showed high responses. This class lay within mean values of the box whiskers plots of more than 200 sediments database data of Applied Aquatic Toxicology research group, HAW-Hamburg.
- Class 3: Elevated critical risk. Consisted of 1% of results of samples (sediments and soils) from Kilombero valley. The samples in this category showed elevated toxic responses in at least two of the bioassays and moderate toxic responses in one bioassay. Such samples lay in the top extreme of 75 percentile of box-whiskers plot of more than 200 sediments database data of Applied Aquatic Toxicology research group, HAW-Hamburg.

Depending on the type of database used and test systems, more than three FRB classes can be deduced (Ahlf & Heise, 2005; Keiter *et al*., 2009). In this study, only three classes were practical owing to low percentage inhibition of bioassays responses and stimulation of *P. subcapitata* bioassay showed by Kilombero valley samples.

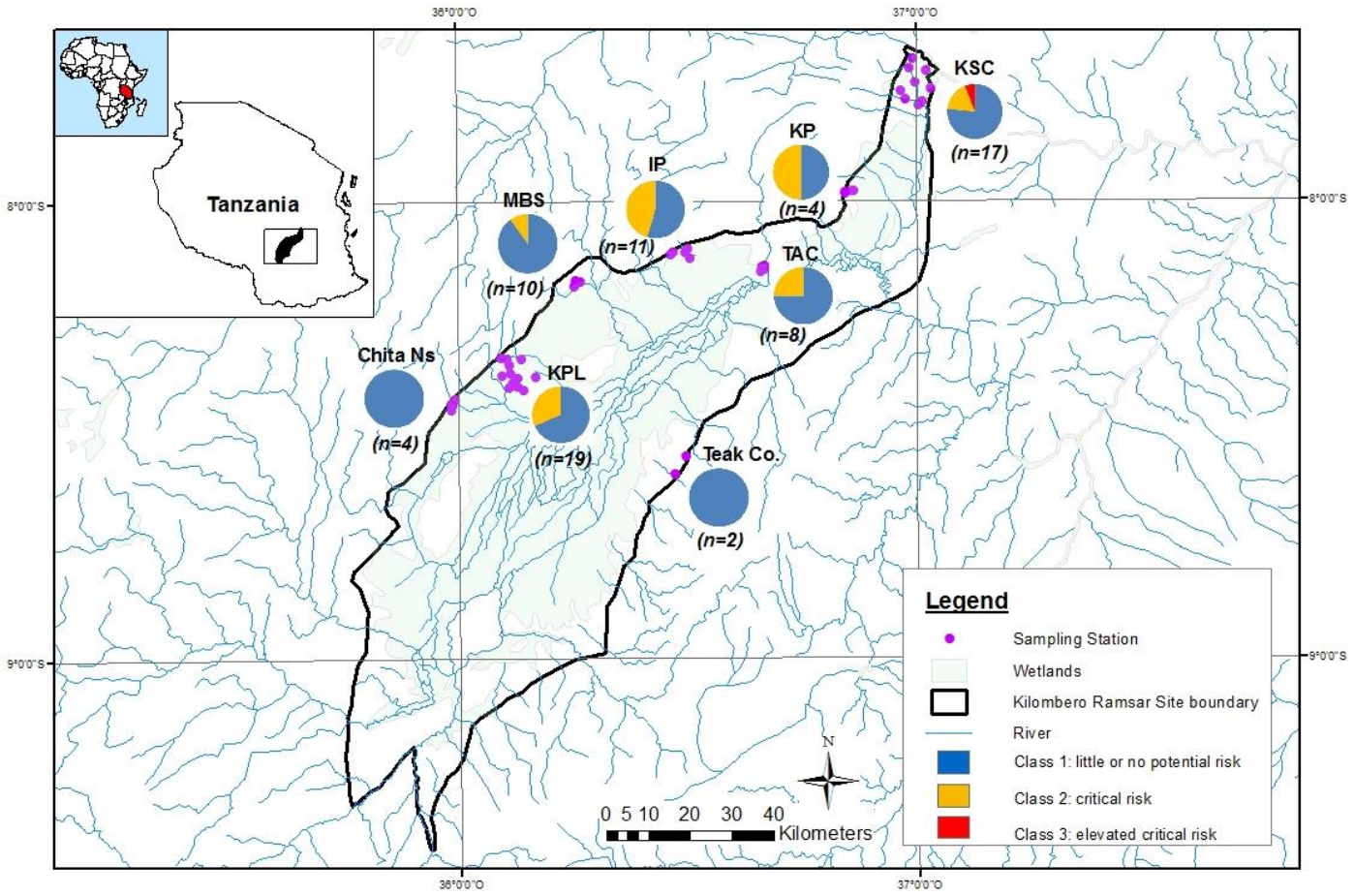


Figure 4.3: A map of Kilombero Valley Ramsar Site with pie charts showing the proportion of bioassay toxicity classes of 75 samples (sediment and soils), for each sampling stations. The sampling stations are marked purple inside the Ramsar site. KSC=Kilombero sugarcane plantations, KP= Kiberege agricultural prison, TAC= Tanganyika Agricultural Centre, IP= Idete agricultural prison, MBS= Mbingu sisters farms, KPL = Kilombero rice plantations, Chita Ns= Chita National service farms, Teak Co = Kilombero valley teak company

The assignment of Chita Ns and Teak Co. into class 1 (Little or no potential risk), is caused by low toxicity shown by the rainy season sediment samples to the three bioassays. Estimated land used for teak plantation (Teak Co) is about 8,000 ha while Chita Ns, had about 80.9 ha used for irrigated and paddy rice production. However, a minimum of two and four samples for Teak Co and Chita Ns respectively, might not be a good representative of these plantations. The number of samples collected in these two plantations was limited during rainy season because of poor accessibility of the agricultural fields, located far apart from each other, and surrounded by thick natural forests/vegetation cover. Nevertheless, from the questionnaire survey, it was revealed that the use of pesticides and other agrochemicals by Chita Ns and teak/wood plantations was very limited or restricted to some plots only depending on the severity of weeds/pests. It was revealed that Teak Company uses more pesticides in the nurseries preparation than in the wood forests. Besides, sediment samples collected from Chita Ns resulted in moderate to elevated stimulation in both *A. globiformis* and *P. subcapitata* bioassays, while those from Teak Co, had shown moderate stimulation in *P. subcapitata* and low toxic responses in *A. globiformis*. This indicates that Chita Ns farms had high nutrient enrichment or organic matter due to the surrounding dense natural vegetation cover.

In contrast, the assignment of FRB class 2 (critical risk) to samples (sediment and soils) collected from MBS, IP, KPL, KSC, KP and TAC among other factors, might be contributed by the presence of pesticide residuals in such samples. For instance, FRB class 2 was assigned into 9 out of 21 samples (sediment and soils), in which pesticides such as diuron, atrazine, glyphosate, AMPA and chlorpyrifos, were detected (Table 3.15). Additionally, these samples had shown moderate to high toxic responses to *V. fischeri* and *A. globiformis* bioassays. Among dry and rainy season samples that showed moderate toxicity, class 2 (critical risk) was largely contributed by dry season samples (soil and sediments). This can be explained by the bioavailability of contaminants during dry season, such as glyphosate herbicides, which are used to clear the farms before subsequent planting seasons. In dry season, there is less effect of surface washout, and therefore, good proportions of herbicides might be retained in the application sites and can only be reduced by solar radiation, volatilization and microbial degradation. Assignment of one sediment sample (from KSC) out of 75 samples (sediment and soils) into class 3 (elevated critical risk) is due to high toxic responses shown by this sample to the three bioassays. The detection of atrazine (0.46 µg/g and glyphosate (0.19 µg/g) in this sample or any other undetected toxicants might have contributed to additive effect of

high toxicities in the three bioassays. This sample was collected in the stream that adjoins effluents from sugar processing plants; therefore, there might be a possibility of other toxicants.

The advantages of using fuzzy logic classification system were considered of importance as described by McNeill *et al*. (1994) in the use of fuzzy logic for system control.

- It requires fewer values to evaluate many variables by using rules, which encompass great complexity for decision-making.
- One can relate output to input, without having to understand all the variables or values, thus permitting more accurate and stable outcome than the conventional data control systems.
- Fuzzy logic rule based systems simplify knowledge acquisition, representation and ultimately help to easy interpretation of data.

4.2.4 Distribution of pesticides residuals in samples from Kilombero Ramsar Site

Depending on the level of cultivation, pesticides are released into the environment in different magnitudes as single substances or as cocktail of compounds to the receiving water streams. Toxicity of pesticide contaminated effluents depends on amount and type of pesticide present in such discharges (Fernández-Alba *et al*., 2001). Prolonged low level release of mixtures of toxic compounds, even lower than species threshold toxic levels, can pose a risk to the ecosystem, because toxicity of such mixtures cannot be linked to single substances effect (Rhind, 2009). In this study, the type of pesticide detected in sample matrices depended on field conditions such as type of crops cycle, sampling season i.e. rainy or dry season, and also on extent of pesticide application. For instance, propoxur and diuron were detected in rainy season water samples, while dry season sediment samples showed high detection frequencies of diuron. Glyphosate and AMPA, which are broad-spectrum systemic herbicide, were mostly detected in dry season sediment, soil and water samples from both rice and sugarcane plantations. This indicates that glyphosate formulated herbicides were much more frequently used during dry season for killing weeds before farm preparation or tilling the land for the succeeding growing season. Atrazine, metribuzin, 2-hydroxyatrazine that are pre and post-emergence herbicides were detected more frequently in rainy season water samples than in sediments. This corresponds well with emergence of weeds during rainy season that lead to an increased use of post-emergence herbicides by sugarcane

plantations. The half-lives of these herbicides in the environment are summarized in Table 4.2. Although both glyphosate and diuron are widely used for weed control, the former was detected (14 times out of 79 dry and rainy samples (sediment, water and soil) from rice farms only, while the latter was detected 12 times out of 57 dry and rainy season samples (water, soil, sediments) collected from sugarcane plantations only. These detection frequencies indicate the distinctions of use of these two herbicides between rice and sugarcane plantations. Besides, sediment and water samples in which glyphosate were detected did not show high inhibition in the *P. subcapitata* bioassay. This could be attributed to its lower concentration in the environmental samples than the lowest effective concentrations. For instance, the highest glyphosate concentration detected in the dry season Kilombero samples (sediment and soils) was 0.55 µg/g, while that of dry season water was 0.05 µg/L, which indicates that although not detected in rainy season water samples, the concentration might even be lower than that due to high dilution rates and its high water-partitioning coefficient (60,000 L/kg), which render glyphosate less mobile in soils (Battaglin *et al*., 2005). However, the reported IC_{50} values on *P. subcapitata,* 96 hours exposure is 5.81 mg $_{\text{acidic equivalent}}$/L at 25±1°C (Tsui & Chu, 2008) which is 10-fold higher than the detected concentrations in the Kilombero samples. Other reported EC_{50} and EC_{10} for *P. subcapitata* exposed to glyphosate (95%) acid, for 48 hours are 270 mg/L and 92.5 mg/L, respectively while for Roundup®- 360 g/L the EC_{50} and EC_{10} (48 hours) are 64.7 mg/L and 13.6 mg/L, respectively (Cedergreen & Streibig, 2005). In an experiment to compare sensitivity of different groups of organisms to glyphosate toxicity, Tsui and Chu (2008) reported that Microtox ® bacteria, *V. fischeri* and protozoans had IC_{50} values between 24.9-29.5 mg $_{\text{acid equivalent}}$ /L, and thus are less sensitive to glyphosate formulations than algae and crustacean. Non-photosynthetic organisms, such as bacteria, are more tolerant to toxicity of glyphosate, because they do not rely on the pathway of aromatic amino acid synthesis, which glyphosate inhibits (Tsui & Chu, 2003). However, a study by Munkittrick *et al*. (1991) indicates that *V. fischeri* is sensitive to a wide range of organic substances and less sensitive to metals. In contrast to the results obtained in this study, samples in which glyphosate were detected showed relatively high toxic responses in *A. globiformis* and *V. fischeri* bioassays. It could imply that other confounding factors, such as high organic nutrient levels and inorganic contaminants (heavy metals, metalloids and radionuclides) that originate from the use of pesticide and phosphate fertilizers, might have contributed to the inhibition of these two bacteria bioassays. Eco-toxicity data indicate that diuron is generally persistent in soil and in water, and its biodegradation products such as DCPMU, 1- (3,4-dichlorophenyl)-3-methyl-urea), DCPU [1-(3,4-dichlorophenyl)urea] and DCA (3,4-

dichloroaniline) are even more persistent in the environment and thus might exhibit higher toxicity to soil and aquatic organisms (Giacomazzi & Cochet, 2004). Diuron is nonvolatile, and its persistence ranges from days to years (Field *et al*., 2003; Okamura *et al*., 2003), which increases its frequency of detection in soils, sediments and in the water samples in both dry and rainy seasons. Propoxur is registered in Tanzania as carbamate insecticide for outdoor control of mosquitoes and other pest insects on agricultural premises. Propoxur (2-isopropoxyphenyl methyl carbamate) is not recommended for use on any food crops. It has a relatively short half-life in water as it degrades within a period of 10 days by photolysis and it takes about 80 to 210 days for aerobic degradation (California EPA, 1997). However, its detection in water samples collected during both dry and rainy season in Kilombero sugarcane plantations might be associated with its potential of use in the sugarcane farms for insects-pests control. Studies by Ma *et al*. (2006a) indicate that EC_{50} for propoxur to biomass population of *P. subcapitata* (freshwater green algae) at 24°C is 2.8 mg/L for 96 hours, while the detected concentration in Kilombero water samples was 0.083 µg/L in combination with other pesticides, which did not show high toxic responses to *P. subcapitata*. During face-to-face interviews with sugarcane plantation agronomists, it was revealed that different mixtures of pesticides were sprayed on the farms for effective pest or weed control. This explains the cause of higher frequencies of detection of various pesticides from sugarcane plantations than from rice plantation. Table 4.2 provides an overview of environmental conditions and degradation half-life of detected pesticide in the soil, sediment and water samples from Kilombero Ramsar Site, which were detected according to different seasonal use patterns. Koc values vary according to soil type, soil pH, acid-base properties of the pesticide and soil organic matter (Weber *et al*., 2004). Koc indicates a tendency of organic contaminants to sorb to soils or sediments, and predicts the mobility of organic soil contaminants. The lower the Koc values of organic chemicals (<1,900 L/kg), the higher their mobility, and vice versa (Wauchope *et al*., 2002).

Table 4.2: Toxicity and degradation half-life under different environmental conditions of detected pesticides

Detected pesticides (Active ingredients)	Degradation half life				Field dissipation	Koc (L/kg) Soil organic carbon-water partitioning coefficient	Toxicity to aquatic test organisms
	Aqueous photolysis	Hydrolysis	Aerobic soil degradation	Anaerobic soil degradation			
diuron	43.1-2180 days (pH 7 at 25°C) [1]	1490 days (25°C, pH 5), 1240-1330 days (pH 7), 2020 days (pH 9)[1]	372 days in silt loam soils[1]	995 days in silt-loam soils[1]	99.9-134 days- on silt clay loam[1] (depends on doses and repeated applications)	418-560[2]	LC_{50} (48hr) = 4.3 to 42 mg/L in fish (depending on *sp*) and 1 to 2.5 mg/L for aquatic invertebrates[1]
glyphosate		>35 days (average over several pH levels and temperatures)[1]	96.4 days (average of five samples with different soil types)[1]	22.1 days in sand-clay loam[1]	44 days (average of two samples with different soil types)[1]	9 to 60,000[3] in a range of agricultural and forest soils (geometric mean for n=28 is 2,072)	IC_{50} (96hr) to *P. subcapitata =24.7* mg $_{AE}$/L)[6], IC_{50} (15 min) to *V. fischeri*=17.5 mg $_{AE}$/L)[6], LC_{50} (48hr) to *C. dubia* =14.7 mg $_{AE}$/L)[6]
propoxur	10 days (pH 7)[4]	16 days (pH 8)[4]	80 and 210 days (pH 7, on silt loam and sandy-loam soils respectively)[4]		-	<1 to 103 High mobility in soils[6]	LC_{50} (48hr) to *P. subcapitata* = 4.01 mg/L[8]
atrazine		>30 days, 25°C, pH 5-9[1]	>146 days in sandy-loam soils[1]	142 days in loam soils[1]	69.8 -102 days in sandy-loam soils[1]	100[9]	IC_{50} (96hr) to *P. subcapitata* =0.026 mg/L[9] EC_{50} (15 min) to *V. fischeri* = 69.4 mg/L[9]
metribuzin		2300-4760 days at (25°C, pH 7-9) [1]	107-172 sandy-loam soils[1]	112-439 days- in sandy-loam soils[1]	39.5 to 138 days in sandy-loam soils[1]	~ 70[9]	EC_{50} (96hr) to *P. subcapitata* =43 µg/L[10]
hexazinone		>56 days, (15°C, pH 5-7)[1]	216 days /stable) in sandy loam soils[1]	>232 days in aquatic sediments[1]	~154 days- in silt loam and ~123 days in loam soils[1]	54[9]	
chlorpyrifos		>72 days, 25°C, pH 5-7 >29 days, 25°C, pH 9) [1]	>179 days in clay soils, >57 days in silt loam soils, 131 days loam-sand[1]	17 days in clay soils and 95 days in loam soils[1]	33-56 days in loam soils (sand-clay-silt)[1]	5300-14800[11]	EC_{50} (30 min) to *V. fischeri*=2.84 mg/L)[12]

[1]Kollman and Segawa (1995), [2]ARS USDA, (2004), [3]Giesy *et al*. (2000), [4] California EPA, (1997), [5]Tsui and Chu (2003), [6]Swann *et al*. (1983), [7]Slabbert and Venter (1999), [8]Wauchope *et al*. (1992), [9]Caux *et al*. (1996), [10]Fairchild *et al*. (1997), [11]Azimi-Gaylon *et al* (2001), [12]Palma *et al*. (2008)

Pesticides with relatively low Koc (less than 1,900 L/kg) such as diuron and propoxur, long hydrolysis and photolysis half-lives, are prone to off-site movement in surface runoff and for permeable soils they can easily leach to ground water. Soil permeability varies according to soil texture from one station to another, for instance silt clay soils are less permeable compared to sandy-loam soils which allows water and other soluble contaminants to percolate through easily (FAO, 2006).

4.2.5 Elemental screening: Risk of heavy/trace metals in the Kilombero valley

Results of metal screening for sediment and soil samples from Kilombero valley, revealed that concentrations of the detected trace metals were in the range of the background concentrations of most surface soils and sediments in various parts of the world (Alloway, 2012) except for thorium (Th) and molybdenum (Mo) whose mean concentrations were higher than the universal reported background levels in surface soils. With the exception of nickel and molybdenum, which were detected only in KSC, IP and MBS, the remaining trace metals were detected in all other stations but in different magnitudes. Principal component analysis (PCA) could not reveal direct relationships between concentration of various metals /trace elements in the samples with detected pesticide or with inhibitions showed in the bioassays. The lack of discrimination in PCA was contributed by having detected the same magnitude of metals concentrations in different samples from different stations.

The mean concentration of thorium (Th) detected in the Kilombero samples (sediment and soil) was 86.29 mg/kg, which is 10-fold higher than its maximum reported natural occurrence concentration (9 mg/kg) in most surface soils (refer Table 4.3). Thorium and other radionuclides in agricultural soils are often associated with the use of phosphatic fertilizers (Hamamo *et al*., 1995; Taylor, 2007). It was revealed in the questionnaire survey that Di-ammonium phosphate (DAP) and *Minjingu* phosphates fertilizers were widely used by all the surveyed rice and sugarcane plantations. Therefore, this explains the reason for elevated mean concentration of thorium in the samples, when compared to natural background levels. In the review study by Lema *et al*. (2014), it is reported that *Minjingu* rock phosphate fertilizers contain a significant level of free radioactive elements such as uranium, thorium, and radium. *Minjingu* fertilizer is locally mined in Tanzania and is widely distributed and used in the country. Moreover, metal based fungicides like copper sulphate, copper oxychloride, and many more that are used, for instance, by teak plantations for wood preservation and by sugarcane plantations to prevent fungal infections, are the potential source of heavy metals enrichment in the Kilombero Valley Ramsar wetlands. However, no samples from these stations or any other sampling sites showed elevated copper concentration than the background levels. This might imply that perhaps the specific sites where such copper-based pesticides were applied were not sampled, and flooding events increases the mobility of contaminants in the area. In the study to determine copper mobility in agricultural fields, Neaman *et al*. (2009) reported that during rain events, dissolved organic carbon might be the major factor

controlling total dissolved copper concentration, while soil pH influences the detection of free Cu^{2+}. Continuous application of metal based pesticides and phosphatic fertilizers in the agricultural fields might contribute to surface soils enrichment of cadmium, zinc, arsenic and radionuclides such as thorium and uranium in the long future. According to Adriano (2001), fertilizers made from magmatic phosphate tend to have negligible concentration of Cd, whereas those from sedimentary phosphate tend to have high levels. Hence, phosphate fertilizers might contain varying amount of Zn, Cd, and other trace elements that originated from phosphate rocks as impurities co-precipitated with phosphate at the time of deposition (Adriano, 2001). The fate and behavior of trace elements depend on their chemistry in soil, i.e. inorganic and organic phases, and their bioavailability which depends on the ambient environmental factors such as pH, organic matter content, clay content, temperature and redox status (Chojnacka *et al*., 2005). Elevated concentrations of heavy metals such as Zn, Ni, Cd, Pb, Mn, Co, Hg, As and Cr might be toxic to most organisms when they are bioavailable in the ambient environments (Kabata-Pendias & Pendias, 1992).Table 4.3 summarizes the concentration range of natural occurrence of some metals in surface soils and the potential concentration range which can pose risk to human or to plants. However, the environmental and human risks associated with such metals depend on their bioavailability. Although within the background levels, the concentrations of nickel, chromium, vanadium and zinc in all sampling stations exceeded the phytotoxic concentration limit. The lack of correlation in PCA between the inhibitions shown by the samples in the *P. subcapitata* bioassay might indicate that the metals were not bioavailable to pose toxic effect on algae.

Table 4.3: Comparison of concentrations of metals/ trace elements from Kilombero samples to the natural occurrence in the environment

Element	Natural occurrence range conc.in surface soils (mg/kg)	Kilombero samples - range conc. (mg/kg)	Phyto-toxicity Conc. (mg/kg)	Potential human health impact conc. (mg/kg)	Comment.
antimony	1 to 4[1]	Not detected	>5[1]	>31[1]	Metalloid which is mobile in the environment and is bioavailable for uptake by plants[1]
arsenic	1 to 55[1, 3]	4.7 to 6.9	>10[1]	Inorganic arsenic is carcinogenic[7]	Primarily associated with Fe[3]
cadmium	0.6 to 1.1[1] 0.01-2.60[3]	Not detected	>4[1]	>37	Cd concentration exceeding 1 mg Cd/kg may occur naturally for soils developed on shale or in sedimentary rocks[3]
chromium	1 to 1,000[1]	97- 279	>1[1]	>30[1] (Cr^{6+}), >10,000 (Cr^{3+})	Behavior and toxicity depends on the variability of its oxidation states
cobalt	6.9-11.3[2] 1-40[4]	2.8 to 23	-	-	Closely associated with manganese in soils[2]
copper	2 to 100[1]	8 to 80	>100[1]	>3,100[1]	Cu is mobile and is a common contaminant in soil and sediments associated with fertilizers and pesticides application
iron	7,000 to 555,000[1]	7868 to 78428	Limited environmental impacts[1].		Most of iron concentration originates from parent rock. High levels interfere with other elements in XRFA
lead	2 to 200[1]	8-32	>50[1]	>400[1]	Binds to soil organic matter and oxides of Fe, therefore remain immobile in soils
manganese	850[4]	178 to 3280			Poorly drained or long time wetted soils might contain high concentration of Mn[3]
mercury	0.01 to 0.3[1]	Not detected	>0.3[1]	>23[1] (Hg) and >6.1[1] (met-Hg)	Highly volatile
molybdenum	0.9 to 1.6[2]	5.4 to 11.9	10-15[5]	-	Most mobile and plant bioavailable in alkaline conditions[2]
nickel	5 to 500[1]	30 to 77	>30[1]	>1,600[1]	Alluvial soils are associated with high concentration of Ni
rubidium	68 to 116[2]	5.3 to 164	-	-	Radioactive element
selenium	0.1 to 2[1]	Not detected	>1[1]	>390	Metalloid that is capable of binding with other toxic heavy metals like Cd, Hg and Ag. Potentially toxic to human at low intakes[5]

Table 4.3 continued

silver	0.01 to 5[1]	Not detected	>2[1]	>390[1] (Carcinogenic)	Rare and precious metal Quite toxic to plants in nutrient solution[6]
strontium	130 to 240[2]	17.2 to 427			Radioactive element, considered most biochemically hazardous to man[6]
thorium	8 to 9.2[2]	42-153			Naturally occurring radioactive metal, associated with phosphate fertilizers[2,5]
titanium	0.33-7038[2]	985 to 8713	>200 mg/kg in plant tissue[6]	Non-toxic[2]	Abundant element, insoluble in water. Physiologically inactive in plants and animals, accumulates in surface soils[6]
vanadium	20 to 500[1]	13 to 186	>2[1]	>550[1] 150-600 mg intake is toxic[6]	Can be associated with Mn, K and organic matter. Highly toxic to soil microbiota[6]
zinc	10 to 300[1]	7.8 to 134	>50[1]	>23,000[1]	Highly soluble, thus a threat to aquatic environment
zirconium	32-850[2]	132-1165	Not adsorbed	Low systemic toxicity[2]	Abundant metal[2]

Sources: [1]USEPA, 2006, [2] (Kabata-Pendias & Pendias, 1992), [3](Traina, 1999), [4] (Levinson, 1974), [5] (Alloway, 2012) , [6]Pais and Jones Jr (1997), [7](Hughes, 2002)

In the absence of human activities in an area, the concentration of detected trace metal contents of the soil and sediment largely depend on that of the rocks from which the soil parent material was derived and on the weathering processes to which the soil-forming materials have been subjected to (Kabata-Pendias & Pendias, 1992). Therefore, since there were no on-going-industrial or mining activities in the Kilombero valley, then the detected metal loads on the agricultural soils and sediment was sum of metal inputs from atmospheric deposition, parent material and from the addition of agrochemicals to the soils. Moreover, flooding and sediment deposition, which occur every rainy season in the Kilombero valley, might enrich the concentration of detected metals in one sampling station to another. Metal-containing particles from various geomorphic or anthropogenic sources might be carried in suspension during flooding and are thus deposited in the alluvial soils of the Kilombero valley. Phosphate fertilizers, pesticides and mineral sewage sludge or animal manures are considered the most important sources of metals such as Cd, As, Cu and Zn contamination in agricultural lands. Appendix 8 presents an overview of worldwide reported concentration of heavy metals and metalloids in commercial fertilizers and animal manures, which has been a source of heavy metals enrichments in agricultural lands.

The concern about anthropogenic inputs of metals to the ecosystem mainly arises from the eco-toxicological impact on plants and soil organisms (Bringmark *et al*., 1998; Palmborg *et al*., 1998), on

aquatic organisms due to surface runoff, and from the uptake via food chains into animal tissues and products, which may results in health effects on animals and humans (Clark, 1992).

4.3 A supplement to a biotest battery: a quest for additional bioassays for samples collected in agricultural areas

The three standardized bioassays used in this study were did not measure herbicides (specifically algae test) or fungicides toxicity. As pointed out earlier, fungicides are also used in agricultural fields, and for Kilombero valley, they were revealed to be used in sugarcane and teak plantations. Since there was insufficient toxicity information from *P. subcapitata* bioassay and no information on fungicides toxicity was available, then the necessity to extend the number of biotests that would be used for risk assessment in agricultural areas was considered important. The development and requirements of new biotests for this study were based on the following criteria: fastness and easiness of the test, the capacity of the biotest to be performed in a classical laboratory and already available devices, and a potential to indicate herbicide or fungicides toxicity in the sample matrices. In the Sections 4.3.1 to 4.3.5 the toxicity information deduced from the biotest battery used to assess the toxicity of Kilombero samples is evaluated and the requirements of bioassays in different fields of application are discussed. The results of two proposed simple and fast bioassays are evaluated in terms of their applicability and suitability in assessing herbicides and fungicides in the samples collected in agricultural areas. These biotests are acute *P. subcapitata* test, which involved measurement of prompt and delayed, fluoresce to assess presence of herbicides in environmental samples, and yeast, *S. cerevisiae* bioassay that was developed in order to assess impact of fungicides in the environment.

4.3.1 Characteristics of bioassays and ecological relevance

The three bioassays used in this study have different biochemical complexity. The use of elutriate (water extracts) in *V. fischeri* and *P. subcapitata* bioassays enables exposure of test organisms to different water-soluble contaminants from the samples matrices. The use of *A. globiformis,* a soil bacterium had an additional advantage of representing organisms that have direct contact with contaminated solids in the environment. Without obstruction of complex biological systems, the toxicity of environmental samples was measured on these low structured organisms by examining biochemical and physiological parameters after exposure to sample matrices. The toxic effects shown by these test organisms would indicate adverse effects on other organisms in the natural environment if the contaminants are bioavailable (Ahlf *et al*., 2002). As shown earlier in Section 3.3,

the toxic responses shown by the three bioassays differed significantly depending on the sensitivity of each test organism to contaminants present in the sample matrices. A battery of bioassays, which uses responses of different test organisms, varied sensitivity, different exposure routes and different toxicity response/end points, is useful for the detection of potential adverse effects of complex mixtures of contaminants (Keddy *et al*., 1995). However, the Kilombero sediment toxicity could be characterized by only two bioassays (*V. fischeri* and *A. globiformis*) without a great loss of toxicity information. *P. subcapitata* showed redundant information on the toxicity of sediment, soil and water samples due to stimulation that could not be explained in terms of toxicity of the samples. *P. subcapitata* results for Kilombero samples limits the suitability of this test for detection of toxic substances in water, or in sediment and soils elutriates of fluvial soils of Kilombero valley. Nevertheless, as shown in Table 3.13, although only 12 samples out of 143 rainy and dry season samples (sediments, soil and water) showed inhibition of *P. subcapitata*, the inhibition responses shown by algae in samples such as KPL11 (15.3%), MBS1 (17.5%), IP2 (20.2%), KSC9 (74.7%), should not be overlooked. Same samples showed either moderate or elevated toxic responses in the other two bioassays (*A. globiformis* and *V. fischeri),* indicating the presence of effective toxicants in the sample matrices. Nevertheless, the results from a battery of bioassays were interpreted together in the fuzzy logic classification system for an integrative ecological risk assessment of the Kilombero valley.

Biochemical variety and complexity of test systems are of great concern during selection of bioassays for ecological risk assessment. For instance, studies by Ghosh *et al*. (1997) found that using three bacterial bioassays (with low biochemical diversities) to assess the impact of 10 pesticides generated the same relative order of toxicities (EC_{50}) of the compounds regardless of the test systems. Conversely, Fernández-Alba *et al*. (2001); Kungolos *et al*. (2009), obtained different toxicity results (EC_{50}) on assessing effects of mixture of pesticides in the test matrices to three different test organisms of diverse complexity and biochemical properties. Table 4.4 summarizes the general requirement of bioassays to various fields of application. Parameters such as ecosystem-relevance, reproducibility, suitability and animal ethics are key issues to consider before using a battery of bioassays to any field of application. If the contaminants of interest have no specific target site in the test organisms, then the bioassay will be less sensitive or not suitable for that particular application.

Table 4.4: General overview of bioassays requirements, profile performance and relevance for various fields of application (modified from Gandrass and Salomons (2001) ,

		FIELD OF APPLICATION						
		Inference or criteria	Environmental law/ Regulations	Effect screening	Early warning system	Bio-monitoring	Research/ teaching	hazard/ risk assessments
GENERAL REQUIREMENTS	Ecosystem relevance	Bioassays results, ecological relevance of test organisms, test design.	+++	+	+	+	+	++
	Sensitivity	Broad spectrum of contaminants	+	+	++	+	-	+
	Reproducibility	Validity of experimental results	+++	+	+	+	+	+
	Standardization	Comparability of test procedures	+++	+	-	-	-	-
	Practicability	Time, space, personnel, cost effective	+	+++	+	+	-	-
	Suitability	Appropriate for target substances	+	+	+	+	+	+
	Public perception	Relevance, understandable to stakeholders	+	-	-	-	-	+
	Speed of response	fast response following exposure	-	+	++	-	-	+
	Automation	Can be run with less or more sophisticated technical equipment at low costs	-	+	+++	+++	-	-
	Animal ethics	According to prevailing laws	+	+++	+	+	+	+

Source: Gandrass and Salomons (2001), - not so important, + important, ++ decisive, +++ very important

Standardization, reproducibility and ecological relevance of the bioassays are considered of importance for regulatory purposes, while animal ethics and practicability of the test systems are of great importance in effect screening (Table 4.4). Different fields of application consider different factors or requirement of bioassays depending on the expected outcome or decision to be made. For instance, requirements such as sensitivity of test organisms and speed of response are left to be decisive because no single species or test organism is sensitive to all substances or contaminants in the environment. Therefore, decision on the type of bioassays will be governed by what is to be

tested, for what purposes, and the characteristics of the contaminants themselves. Other parameters such as standardization speed of response, automation etc., might not necessarily be of importance for all fields of applications. The most challenging requirement of bioassays is ecosystem-relevance due to complexity and limited understanding of the chemical interaction with various components of the ecosystem. Ecosystem relevance means that results of biological testing are of importance for the understanding of the general effects of a certain substance to an ecosystem (Calow, 1991). Nevertheless, the other requirements can be fulfilled to a certain satisfaction at different levels of test systems (Gandrass & Salomons, 2001).

4.3.2 AAT: delayed and prompt fluorescence measurement

The objective of these experiments was to further evaluate the reason of *P. subcapitata* stimulation in the chronic 72-hr test. An assumption based on the observed stimulations in chronic *P. subcapitata* was developed and tested in acute tests (30 minutes). The hypothesis that perhaps the stimulation of measured algae auto-fluorescence was due to blockage of continuous electron flow in Photosystem II by chemicals or pesticides that interfere with the electron transport system, such as phenyl-urea herbicides was tested. *P. subcapitata* was exposed for 30 minutes to four test chemicals namely photosystem II inhibitors (diuron and, isoproturon) and chemicals with different mode of actions (3,5-DCP and glyphosate). Delayed and prompt fluorescence were measured and the pattern of response evaluated. Results showed that PS II inhibitors resulted in inhibition in DF and stimulation in PF, in contrast to chemicals with different mode of actions (Table 4.5).

Table 4.5: Pattern of DF and PF after 30 minutes exposure of P. subcapitata to 3,5-DCP, glyphosate, diuron and isoproturon.

Test chemical	PS II inhibitor?	Prompt fluorescence	Delayed fluorescence
3,5-DCP	NO	Inhibition	Inhibition
Glyphosate	NO	Inhibition	Stimulation
Diuron	YES	Stimulation	Inhibition
Isoproturon	YES	Stimulation	Inhibition

According to Eisentraeger *et al*. (2003), *in vivo* chlorophyll fluorescence measurements offer a wide range of physiological studies of living cells with active photosynthesis. Delayed fluorescence (DF) results from back reactions in the photosystem II (PS II) and its emission ceases within few microseconds to seconds after illumination (Berden-Zrimec *et al*., 2007; Jursinic, 1986). In order to detect such electrons, in this study, DF was measured in an integration and lag time of 80 and 100 microseconds, respectively, after illumination. DF is related to trapped electrons in macromolecules in the photosystem II cytoskeleton (Scordino *et al*., 2000), thus useful for estimating photosynthetic activity or the structural state of algae, and can be used to evaluate effects of toxicants due to fluorescence inhibition (Drinovec *et al*., 2004). Studies by Berden-Zrimec *et al*. (2007) and Katsumata *et al*. (2006) advocate that short-term inhibition of DF emission (i.e. 15 minutes to 1 hour) after exposure to a toxic chemical can be correlated with long-term growth inhibition (i.e. 72 hours) in green algae.

In contrast, prompt fluorescence (PF) is sensitive to inhibitors of photosystem II centers, and its emission after absorption of light by chlorophyll ceases immediately, within nanoseconds, when the excitation illumination is terminated (Eullaffroy & Vernet, 2003; Schreiber *et al*., 2002). In this study, both lag time and integration time were maintained at zero for PF measurements. According to Drinovec *et al*. (2004) and Katsumata *et al*. (2006), prompt light emission is rapidly quenched and therefore, toxic information on reactions that are distant from the PS II or PS I center (e.g. pQ pool, proton gradient, and NADPH) may not be freely available.

The contrasting responses of algae to diuron and isoproturon herbicides observed in this study (Table 4.5), i.e. inhibition of growth rate in delayed light emission and stimulation in prompt light

emission might be due to their specific mode of actions, as shown earlier in Fig. 3.14, which block the electron transport systems. The trapped or blocked electrons in the macromolecules of the PS II might have contributed to observed stimulation of prompt fluorescence. Although in different experimental setting, the same pattern of delayed and prompt fluorescence responses by PS II inhibitors was shown in various studies (Jursinic, 1986; Katsumata *et al*., 2008; Leunert *et al*., 2013; Scordino *et al*., 2000). The inhibitory response in the DF by the same phenyl-urea herbicides (diuron, isoproturon) might be due to diminished electron flow that led to decline of the intensity of emitted delayed light. For chemicals or herbicides whose effects are not on the electron transport system like 3,5-dichlorophenol and glyphosate, the PF and DF do not show a clear contrasting pattern. Glyphosate interferes with aromatic amino acids synthesis by inhibiting 5-enylpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme that catalyzes the reaction for some amino acid synthesis in plants and bacteria cells (Dill, 2005). It results in the elevation of glyoxylate enzymes, which in turn inhibits RuBP carboxylase enzyme responsible for carbon fixation (Ma *et al*., 2001; 2003). 3,5-dichlorophenol uncouples oxidative phosphorylation in mitochondria by inhibiting the coupling between the electron transport and phosphorylation reactions, an effect which inhibits ATP synthesis, and oxygen reduction (Terada, 1990).

Moreover, glyphosate results in stimulation of DF while 3,5-dichlorophenol showed inhibition in the acute algae test (Table 4.5). According to Murakami *et al*. (1975) the delayed light is attributed to back-reactions between the reduced primary acceptor (Q^-) and the oxidized primary electron donor (Z^+). Any condition that favors the recombination of Q^- and Z^+ also enhances the delayed-light emission. The back reaction is enhanced by a positive membrane potential and a higher proton concentration in the inside than on the outside of the thylakoid. A healthy chloroplast membrane structure is necessary for the separation of positive and negative charges and their stabilization for energy production during photo-phosphorylation. Therefore, any damage to the thylakoid membrane by toxicants or addition of un-couplers of phosphorylation to the test matrices such as 3,5-dichlorophenol will cause an increase in thylakoid membrane permeability to H^+ (Fig. 4.4).

Moreover, salts and weak acid based toxicants, like glyphosate which is a weak acid, can cause stimulation of delayed light emission due to a reduced primary electron acceptor and oxidized donor of photosystem II located on the outside and inside of the thylakoid membrane and thus the reaction becomes a pH-dependent equilibrium (Murakami *et al*., 1975). Stimulation in DF by glyphosate therefore, might be due to the generation of a potential difference across the thylakoid, caused by a difference in cation permeability that tends to depress light emission.

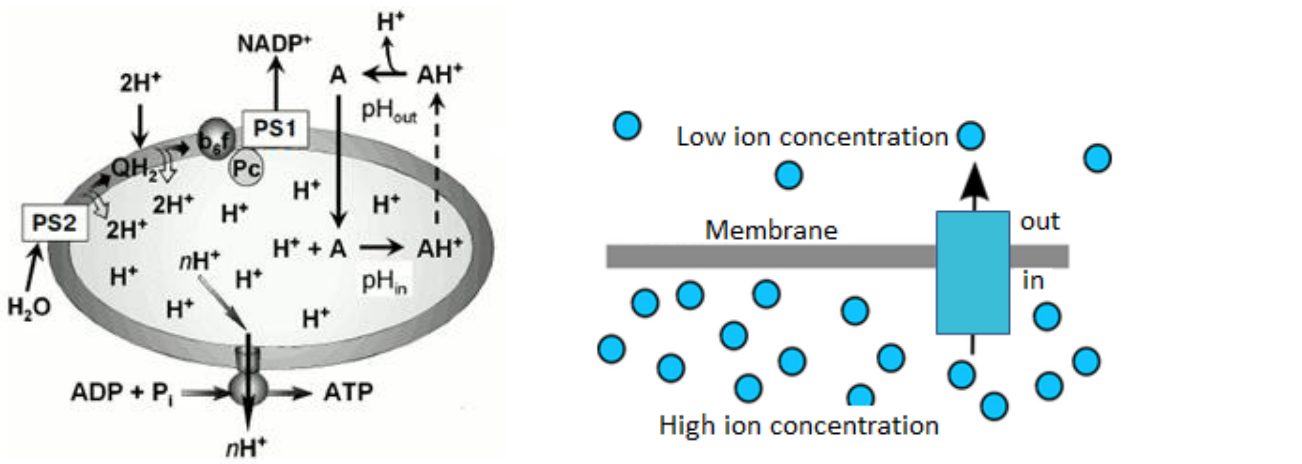


Figure 4.4: Membrane potential across the thylakoid during photosynthesis process, modified from Tikhonov (2012)

The observed pattern of results demonstrates that the presence of photosystem II inhibitors in the test matrices might contribute to stimulation of emitted prompt fluorescence and inhibition of delayed light when measured under acute tests. Therefore, if chronic *P. subcapitata* bioassay is used in combination with the acute algae bioassay, it might be useful in providing further information to identifying priority areas or hotspots for further investigation of pesticides contamination. A few researchers (Goltsev *et al*., 2009; Katsumata *et al*., 2006) have explained the features of delayed fluorescence in algae, *P. subcapitata* that can be used for risk assessment.

4.3.3 AAT-Responses to environmental samples supplemented with pesticides

Different concentrations of photosystem II inhibitors (diuron, isoproturon) and non-photosystem II inhibitors (glyphosate and 3,5-dichlorophenol) were added to elutriates of sediment samples (from Elbe River estuary), which had shown less than 10% and more than 80% inhibition of prompt fluorescence in the conventional *P. subcapitata* (72 hours) bioassay. The objective was to assess whether it would be easy to identify the effect or interference of sample matrices in the acute tests to the pattern of response shown by photosystem II inhibitors when they are in pure water. The results are summarized in Table 4.6.

Table 4.6: Summary of overall algae DF and PF responses to the test chemicals in water and in sample elutriates

Test chemical	Matrix	Response		Mechanism of action
		PF	DF	
diuron	Water	+ +	+/-	Phenyl-urea herbicides, inhibitors of photosystem II (PS II)
	Samples	+ +	- -	
isoproturon	Water	++	+/ -	
	Samples	+ +	-/+	
glyphosate	Water	+	++	Inhibits EPSPS a key enzyme for amino acids synthesis
	Samples	+	+	
3,5-DCP	Water	+	+	Uncouples oxidative photophosphorylation
	Samples	+	+/-	

+ indicates <50% stimulation, *++* means >50% stimulation, - means <50% inhibition, - - means >50% inhibition, *+/-* lower concentration resulted in stimulation while higher concentrations showed inhibition, *-/+* inhibition is dominant over stimulation

The observed stimulation in PF and DF by almost all concentrations of glyphosate and 3,5-DCP when added to samples might be due to influence of physical parameters, such as organic nutrients, of samples matrices. When isoproturon and diuron were added to samples matrices, the extent of response differed from one sample to another, but the pattern of inhibition of DF and stimulation of PF was clearly pronounced in one sample (SH-3), which showed extreme high inhibition (more than 80%) of PF in the 72 hours test. However, no complete inhibition response was observed in sample SH-2, which also had a high inhibition (more than 80%) in the 72 hours test, as compared to when diuron and isoproturon concentrations stand alone in pure water. The inhibition of PF by samples SH-2 and SH-3 (samples that showed high inhibition of more than 80% in chronic test) was reversed to stimulation of PF and inhibition of DF in the acute *P. subcapitata* tests, when photosystem II inhibitors were added to such samples. Environmental samples contaminated with low concentration of PS II inhibitors or phenyl-urea herbicides like diuron and isoproturon, showed the same trend as observed when tested in pure water. Therefore, for quick screening purposes, DF and PF measurement under acute algae tests can be used to identify the presence of PS II inhibitors in environmental samples collected in areas surrounding agricultural fields. However, different pattern of response might be observed under acute *P. subcapitata* tests due to interference of physical-chemical properties of the environmental samples under concern. The conclusion which is derived from acute algae test is that it is possible to fast screen for the pattern of response using DF and PF measurement, for elutriates of samples collected from agricultural or pesticides contaminated areas, but not much information could be confirmed while using chemicals with different mode of action, as shown by glyphosate and 3,5-dichlorophenol, which showed stimulation in both PF and DF, while in sample elutriates and in pure water.

Kilombero samples

Furthermore, DF and PF were measured in 24 Kilombero samples (sediment and soils). 15 out of 24 samples had shown stimulation (from 30% to 100%) and maximum inhibition (74%) in the 72-hr algae test, and 14 out of these 24 samples had pesticides like diuron, glyphosate, atrazine, and AMPA detected in them. Results were that different samples showed different patterns of response in both DF and PF. For instance, samples where diuron was detected did not necessarily show the trend of stimulation in PF and inhibition in DF when measured under acute tests. However, the pattern observed in the samples was related to the presence of chemicals which might have the same mode of action as 3,5-DCP or as glyphosate, although there were no direct associations with the samples where glyphosate was detected. The absence of direct correlation between the stimulation responses observed in chronic 72 hours algae test and the pattern observed in acute tests or the presence of detected pesticides might be due to influence of organic nutrients, or presence of other type of chemicals, or fluorescent substances or algae species which emit light at different wavelengths when illuminated by monochromatic light. Different species of phytoplankton in natural waters, for instance, emit delayed light at different wavelengths (Bodemer, 2004). Moreover, emissions from sample matrices may lead to quenching effect between electron donor and acceptor, and thus lead to interference from of emitted fluorescence, which might not change over the short period of acute bioassay. This effect of sample matrices interference has been documented in animals fluorescence immune assays, where antigens are used to reduce the quenching effect during fluorescence polarization (Wild, 2013). However, while in this study DF was measured at an integration time of 100 µs, some studies have reported that long-term DF emission (measured for seconds or minutes) might prevent interference problems with fluorescent backgrounds in natural samples (Istvanovics *et al*., 2005).

4.3.4 Fluorescence intensity and cell density: 72-hr algae growth inhibition test

The objective of this experiment was to assess whether there is a correlation between the emitted algae auto-fluorescence (PF) or observed stimulation and actual cell density in the test medium, when *P. subcapitata* was exposed (for 72 hours) to different concentrations of PS II inhibitors (diuron and isoproturon) and chemicals with different mode of action (3,5-DCP, glyphosate). The results showed that the emitted algae auto-fluorescence and cell density of *P. subcapitata* decreased with concentration of test chemicals (3,5-DCP, glyphosate, diuron, and isoproturon). Only the two lowest concentrations, 0.2625 mg/L and 0.525 mg/L of glyphosate resulted in 14% and

4% stimulation of algae auto-fluorescence, respectively. The rest of test concentrations of glyphosate, diuron, isoproturon, 3,5-dichlorophenol resulted in inhibition of *P. subcapitata.* The inhibition by test chemicals as compared to stimulation result in from the Kilombero samples might be due to toxicity imposed by such chemicals in the absence of interference of organic nutrients in the test matrices. Moreover, the inhibition might have resulted from pH changes in the test media due to acidic or alkalinity of the test chemicals (Cedergreen & Streibig, 2005; Fahl *et al*., 1995) after 72 hours of incubation with formulated products. Commercial forms of pesticides might be highly acidic or alkaline, therefore under high environmental concentrations of herbicides, like glyphosate (e.g. following application), transient effects due to acidification might prevail, which can lead to under or over estimation of toxic response (Pereira *et al*., 2009).

Higher fluorescence unit per cell (about two-fold) in the test concentrations than in the controls were observed in all concentrations of glyphosate, 3,5-DCP concentrations lower than 8.0 mg/L, and in diuron concentrations lower than 0.56 µg/L. This indicates that the inhibition of prompt fluorescence by these test concentrations to individual cells was more pronounced than overall fluorescence inhibition on group of algae cells. Besides, the emitted fluorescence per individual cell could not directly be correlated with the increased or decreased isoproturon concentrations because it fluctuated among different concentrations. Therefore, from this experiment it was concluded that emitted fluorescence per well might be enhanced or interfered by light scattering from algae cells and sample matrices. However, per cell fluorescence may give extra information and can be used to distinguish florescence due to algae cells and fluorescence produced by presence of fluoresce particles or bacteria in the sample matrices, especially during measuring DF and PF under acute test. Therefore, calculating per cell fluorescence in acute PF and DF measurement is recommended in order to get a broader overview of fluorescence emitted due to back reactions and that emitted by active photosynthetic cells in environmental samples. In vivo fluorescence spectra that measure fluorescence per individual cell have been successfully used in discriminating algae species in environmental sample matrices (Determann *et al*., 1998; Hilton *et al*., 1989).

4.3.5 Yeast, *S. cerevisiae* bioassay

This bioassay was developed based on (Fai & Grant, 2009a) and optimized in our laboratory in order to assess eco-toxicological impact of fungicides in the environment. The optimized parameters were tested on three fungicides and on Kilombero samples.

4.3.5.1 Optimization of resazurin concentration and incubation period with fungicide

S. cerevisiae was exposed (from 1 hour to 4 hours), to different serial concentration of copper sulphate up to a maximum concentration (5 mg/L). The concentration of copper sulphate lower than its reported EC_{50} of 20.7 mg/L (Fai & Grant, 2009b) were used in order to examine the trend and viability of *S. cerevisiae* to reduce resazurin when exposed to low concentration of environmental stressors. Different range concentration of resazurin was used in order to establish nominal concentration that would produce feasible resorufin fluorescence intensity after exposure to yeast cells. Results indicated that with an exception of 20 mg/L of resazurin in 4 hours incubation period, highest inhibitions of 50 ± 5% were observed during 2.5 hours and 4 hours of exposure of yeast cells to the highest concentration (5 mg/L) of copper sulphate at all resazurin test concentrations (Fig. 4.5).

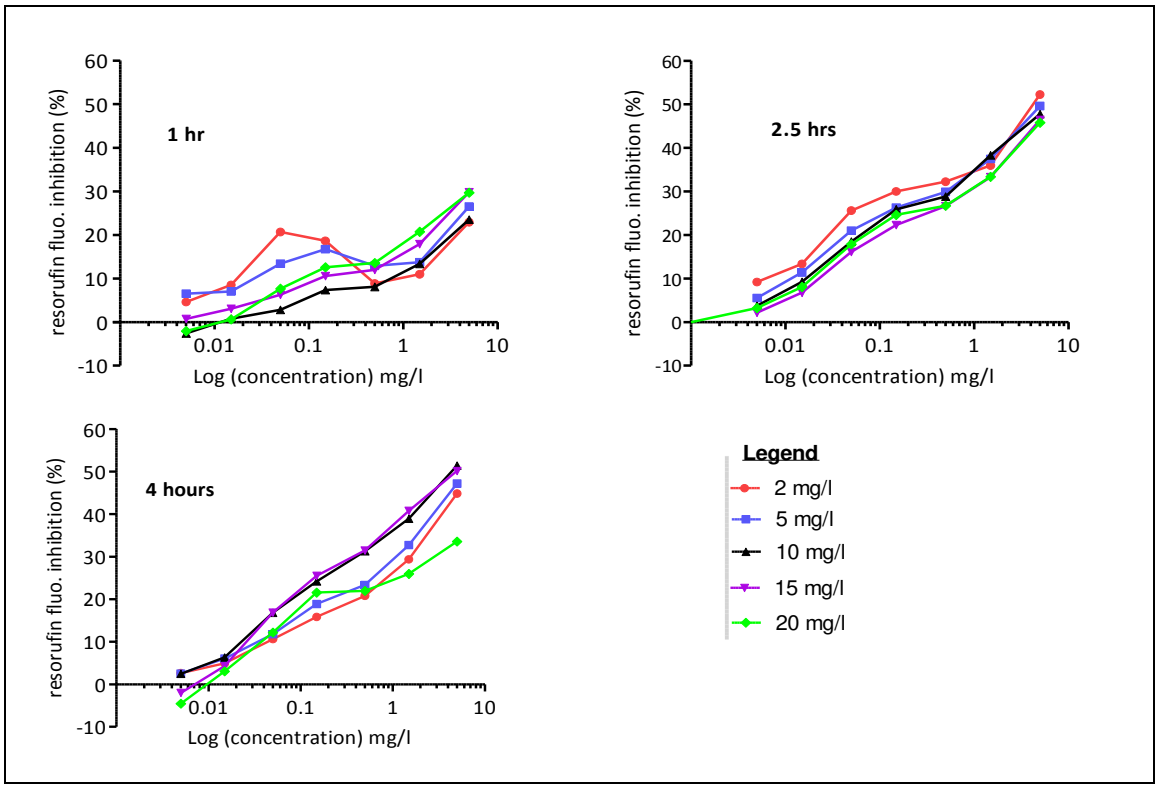


Figure 4.5: Relationship between resorufin fluorescence inhibitions tested by different resazurin concentrations after exposure of S. cerevisiae (up to 4 hours) to different concentrations of copper sulphate.

One-hour incubation was considered not useful because of the observed fluctuations of resorufin inhibition, especially in the highest concentration of copper sulphate (Fig. 4.5). The fluctuation responses might have resulted from the struggle of yeast cells to acclimatize into a test medium during the first 1 hour of exposure. 4 hours incubation of *S. cerevisiae* with fungicide, and resazurin concentrations (10 mg/L and 15 mg/L) were considered sufficient to show at least 50% resorufin fluorescence inhibition after exposure to 5 mg $CuSO_4$/L. The assumption was that concentration of $CuSO_4$ higher than 5 mg/L would show resorufin inhibition of more than 50% when using either 10 mg/L or 15 mg/L of resazurin nominal concentrations. However, only the former was favored, since they both showed the same magnitude of inhibitory response. With this low concentration of copper sulphate, no IC_{50} could be attained. Thus, 10 mg/L resazurin nominal concentration was considered economical and sufficient to show resorufin fluorescent signal, especially when the yeast cells were exposed for at least 4 hours to copper sulphate concentrations higher than 5 mg/L, which was used during optimization.

4.3.5.2 Exposure of *S. cerevisiae* to different fungicides

S. cerevisiae was exposed (from 1 hour to 24 hours) to different concentrations of three fungicides: prochloraz, thiabendazole and copper sulphate. Inhibition was calculated in comparison to negative controls. Results indicate that yeast cells showed different toxic responses to copper (II) sulphate, prochloraz, and thiabendazole fungicides over a varying period of exposure, because of different modes of action of these test chemicals. For instance, copper (II) sulphate inhibits multi-site of different enzymes of yeast; while prochloraz inhibits ergosterol synthesis, and thiabendazole interferes with nucleic acid metabolism and protein synthesis of fungi (Fai & Grant, 2009b). Higher concentrations of inorganic copper (II) sulphate affected the viability of yeast cells during short-term exposure up to 3 hours, while concentrations lower than 15 mg $CuSO_4$/L resulted in stimulation (Table. 3.24). The observed stimulation of resorufin fluorescence response to low concentrations of copper (1 – 10 mg/L), and in almost all concentrations of organic fungicides prochloraz and thiabendazole can be inferred to as hormesis, a stimulatory effect caused by low levels of potentially toxic agents (Stebbing, 1982; 2002), whereby the capacity of the *S. cerevisiae* cells to adapt to the system increases with exposure period, but when the exposure period and concentration exceeds the threshold level, the system becomes overloaded thus resulting to inhibition. Same stimulation responses were observed on growth rates of fungal *Laomedea flexuosa* hydroid colonies exposed to low concentrations of copper, Cu^{2+} (Stebbing, 2000; 2002). A number

of studies conducted at varying experimental set up and different end points determination have shown the same trend of stimulatory effect of low levels of toxic substances on growth rate of test organisms (Cook & Calabrese, 2006; Stebbing, 1982) and an inhibitory response in higher concentrations of a toxicant due to inability to recover from stress (Calabrese, 2008; Calabrese & Baldwin, 1997). However, the reason for the observed stimulatory effect by prochloraz and thiabendazole could not be explained in this test, therefore, only results from copper sulphate were considered reliable and reproducible among different tests and replicates. Thus, copper sulphate was used as a positive control while using environmental samples. When the tests were conducted in different days and different plate configuration, prochloraz and thiabendazole showed high stimulation up to 100% and variation among replicates were quite high, up to 12% (results not shown). Therefore, the results could not be considered reliable because of over stimulations and no dose response curve could be attained; however, further experiments to test effects of these and other organic fungicides on *S. cerevisiae,* in relation to their mode of action is recommended

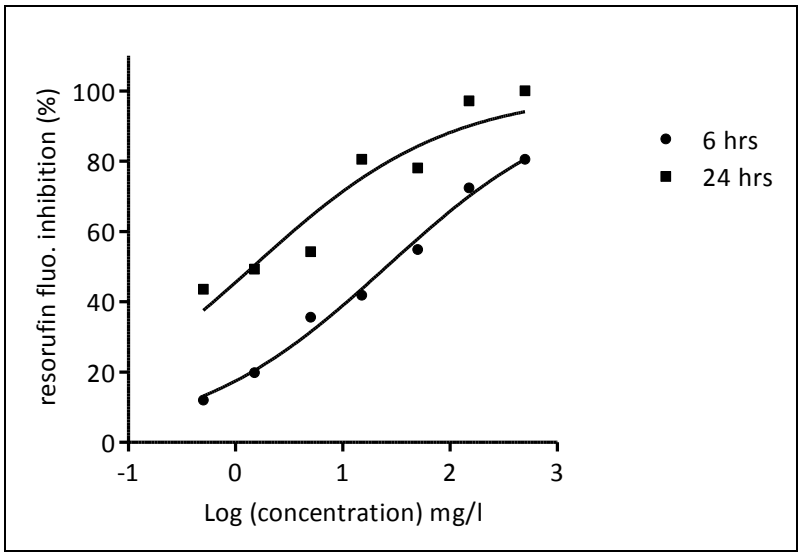


Figure 4.6: Dose-response curves of percentage inhibition of resorufin fluorescence after exposure of S. cerevisiae (6 hours and 24 hours) to different concentration of copper sulphate. (Resazurin concentration used 10 mg/L)

In all incubation period of *S. cerevisiae* (up to 3 hours) with copper sulphate, the resorufin fluorescence was stimulated. Inhibition response started to be observed at 50 mg $CuSO_4$ /L three hours after exposure to copper sulphate. The dose response curve could be attained from 6 hours of exposure to copper sulphate (Fig. 4.6). The calculated IC_{50} after 6 hours and 24 hours exposure were 25.58 mg/L and 1.46 mg/L of $CuSO_4$, respectively. Since the 6 hours IC_{50} (25.58 mg/L) obtained

from this preliminary experiments was slightly higher than the IC_{50} of 20.7 mg/L of copper sulphate reported in literature (Fai & Grant, 2009b), then it was assumed that at least a minimum of 4 hours of incubation of yeast cells with the concentrations of copper sulphate used in this study, would show feasible responses, without depriving the yeast cells for essential nutrients to grow during experimental set up.

Therefore, optimized important parameters during *S. cerevisiae* bioassay development were nominal resazurin concentration (10 mg/L), *S. cerevisiae* starting cell density ($O.D_{600}$ = 0.5± 0.01), length of incubation of test matrices with fungicides (4 hours) and with resazurin (40 minutes). The choice of starting yeast density and length of incubation of yeast with resazurin might have a significant impact in the anticipated end points. Yeast cell density in the test-wells should be proportional to sufficient nutrients for growth during the entire period of incubation with test chemicals or with environmental samples (Button, 1985). Resazurin (blue non-fluorescent) is an oxidation–reduction indicator used to evaluate cell growth (McNicholl *et al*., 2007). All respiring cells are capable of reducing resazurin to resorufin (pink and highly fluorescent) which can further be reduced to hydro-resorufin (colorless and non-fluorescent) (O'Brien *et al*., 2000). In this study, resazurin was dissolved in buffered saline (1X PBS) of adjusted pH 7.5 using MOPS (pH 8.2) in order to maintain higher pH for less reduction of resorufin when measurement were taken. Resorufin fluorescence is strongly reduced at lower pH, while at pH above 7.5 both resazurin and resorufin are in their anionic forms, and resorufin is highly fluorescent (Bueno *et al*., 2002). Therefore, it is important to maintain a high pH in order to reduce uncertainties of underestimating the reduction resulted from viable cells and those resulting from low pH of the test matrices. However, longer incubation period (than 40 minutes) of resazurin with high yeast cell densities might result into an extensive reduction of resazurin by metabolically active cells in the test matrices, which can lead to colorless product and hence underestimation of cellular activity. The same (40 minutes) incubation period was used by Fai & Grant (2009) in order to give sufficient time for resorufin fluorescence detection.

If this simple, fast and easy *S. cerevisiae* bioassay is optimized and standardized, it can be a suitable bioassay for ecotoxicological assessments of fungicides in environmental samples collected from agricultural areas or from areas prone to fungicide contamination. Although this yeast test, *S. cerevisiae*, is not standardized, it has been used successfully in various cell proliferations and

ecotoxicological studies. For instance, (Fai & Grant, 2010; Fai & Grant, 2009b) advocate the use of *S. cerevisiae* in ecological risk assessment.

4.3.5.3 Exposure of *S. cerevisiae* to Kilombero soil-sediment–water samples

The optimized test parameters were used to assess impact of 64 Kilombero dry season samples (sediment soil and water) on yeast cells. *S. cerevisae* were exposed for 4 hours to sediment/soil elutriates and water, and 40 minutes in 10 mg/L resazurin. Inhibition of *S. cerevisiae* metabolic activity was calculated in relation to negative controls. *S. cerevisiae* showed overall same magnitude of inhibition to the sediment, soil and water samples as in *A. globiformis* and *V. fischeri* bioassays. A few water samples showed extreme low (stimulation 7%) or high inhibition (maximum 52%), but the observed mean resorufin fluorescence inhibition by sediment, soil and water samples were in the same magnitude of 13.41%, 16.32% and 16.09%, respectively. This indicates that *S. cerevisiae* was equally sensitive to dissolved contaminants in water, as well as in soil and sediment elutriates. In the cluster analysis, with an exception of *P. subcapitata* bioassay, *S. cerevisiae*, *A. globiformis* and *V. fischeri* were closely associated to each other and to the sample parameters such as salinity, pH, and C/N ratio. This might imply that bacteria and fungi metabolic activities might be inhibited by the same contaminants in the environmental samples. For instance, samples where glyphosate and AMPA herbicides were detected showed elevated inhibition in the *S. cerevisiae* bioassay. It implies that perhaps *S. cerevisiae* is also affected by the presence of herbicides in the matrices. Eckel *et al*. (2008) showed that herbicides which inhibit basic cellular functions rather than the plant photosynthesis target, e.g. sulfonylurea, have a potential to inhibit growth rate of yeast, *S. cerevisiae* on minimal media. Such herbicides have the ability to inhibit the enzymes responsible for amino acid synthesis in bacteria and in fungi (Eckel *et al*., 2008). The inhibition or stimulation responses of *S. cerevisiae* to the sample matrices could not, however, directly be linked to the presence of fungicides in the samples, because screening of fungicides was not performed in this study.

This study found that apart from bacteria and algae bioassays, *S. cerevisiae* bioassay can also be used as a tool to identify contaminated hotspot in the areas surrounding agricultural fields. A variety of fungicides are used in Kilombero valley teak plantations for wood preservation and in sugarcane fields to prevent sugarcane-stem fungal infections. Bünemann *et al*. (2006) showed that fungicides are more toxic to soil organisms than herbicides or insecticides and if used in large quantities, have a great potential to affect beneficial soil fungal species.

4.4 Kilombero valley: Human and environmental risks of pesticides

One of the major challenges of agrochemicals use worldwide is on how to reap the benefits of such chemicals, while at the same time avoiding their harmful effects to humans and to the environment at large. Hazard identification or quantification and pattern of use of a good number of classes of pesticides have been studied for decades in developed world. That has even led to a ban on the use or production of certain active ingredient of pesticides in such countries where are used or produced. Extensive studies, literature and data on fate of pesticides in the environment and effect to ecosystem and to human health are widely available for regulatory bodies worldwide and to public. In this study, the following groups of pesticides were detected in both dry and rainy season samples (water, surface sediments and soils), surrounding sugarcane and rice plantations. Such pesticides were, o*rganophosphorus* (chlorpyrifos, glyphosate, AMPA), *triazines* (atrazine, 2-hydroxy-atrazine), *triazinone* (metribuzin, hexazinone), *phenyl-urea* (diuron, monuron, desmethyldiuron) and *carbamate* (propoxur). Samples collected adjacent to pesticide mixing points, tanker filling, or equipment-washing streams resulted in more frequencies of detection of pesticides and a few showed elevated toxic responses in one or two of the three bioassays used in this study than samples collected elsewhere. The risks of pesticides in the ecosystem depend on the characteristics of a chemical itself and on the level and pattern of exposure. Generally, the crucial issue for Kilombero valley which was revealed during field surveys and sampling were unsafe handling practices of pesticides, for example, pesticide spillage at retail marketing places, spillage during filling up the sprayers, cleaning of the spraying equipment in open waters, on-farm disposal of the pesticide containers and mixing pesticides close to streams, wetlands and ditches where contamination of other areas of the Ramsar ecosystem was susceptible. Lack of clean water or running from tapes is a big problem for Kilombero valley, as with many remote areas of Tanzania. During field surveys and sampling it was revealed that households that live in the remote villages of Kilombero valley, where big plantations are located, depended on the neighboring wetlands, shallow wells, rivers or streams to provide water for domestic purposes and fishing. When associated with agricultural drains, it contributes to potential of human health risk especially poor households that border the surveyed sugarcane and rice plantations. For instance, there was shallow water well within one of the rice farms, which was used by the neighboring poor villagers to get water for domestic purposes. Glyphosate and AMPA were detected in the sediment samples collected from this well, and both its water and sediment elutriate showed high toxic response to *V. fischeri* (51.2% inhibition) and little toxic response (7% inhibition) in *P. subcapitata* bioassays, while

sediment slurries showed moderate toxic response to *A. globiformis* (25% inhibition). Moreover, in another rice plantation, it was also observed that water which drains from paddy rice was ignorantly used by the surrounding communities for domestic purposes, children were swimming in the stagnant waters, and even fishing for household sustenance. Therefore, continued use of water that drains from agricultural fields or consuming contaminated fish might lead to long term human health impacts. However, these poor and unhygienic sources of water are very common during dry season, when most of accumulating waters in the wetlands and river tributaries are dried out.

As described by Mackay *et al*. (1997), three factors which control the fate of chemicals in the environment are the prevailing environmental conditions, properties of the chemical itself that influences partitioning and reaction tendencies, and the pattern of use i.e. into which environmental compartment the chemical is introduced, how and in case of pesticides with which additives the active ingredients is applied. For instance, when compared to aqueous layer or surface sediments, bottom sediments are exposed to less oxygen, little or no photolysis, which might render the chemicals less bioavailable due to increased sorption rates (Mackay *et al*., 1997). However, benthic organisms are prone to contaminants in the bottom sediments, which also might become bioavailable to aquatic organisms like fish and phytoplankton when re-suspended. Kilombero valley is prone to flooding during rainy season (March to June), extended days of solar radiation with strong intensity, high relative humidity >80%, and maximum annual temperatures ranging from 28°C to 35°C-typical for tropical climatic pattern. These tropical weather conditions accelerate the distribution of pesticides in the environment. In general, environmental and human risks of pesticides depend on three main aspects, environmental factors that accelerate the dissipation of pesticides, which is favored by characteristics of a pesticide in concern, handling, and management issues as outlined in Fig. 4.7 and more detailed in the previous Sections 1.3.4 and 1.3.5.

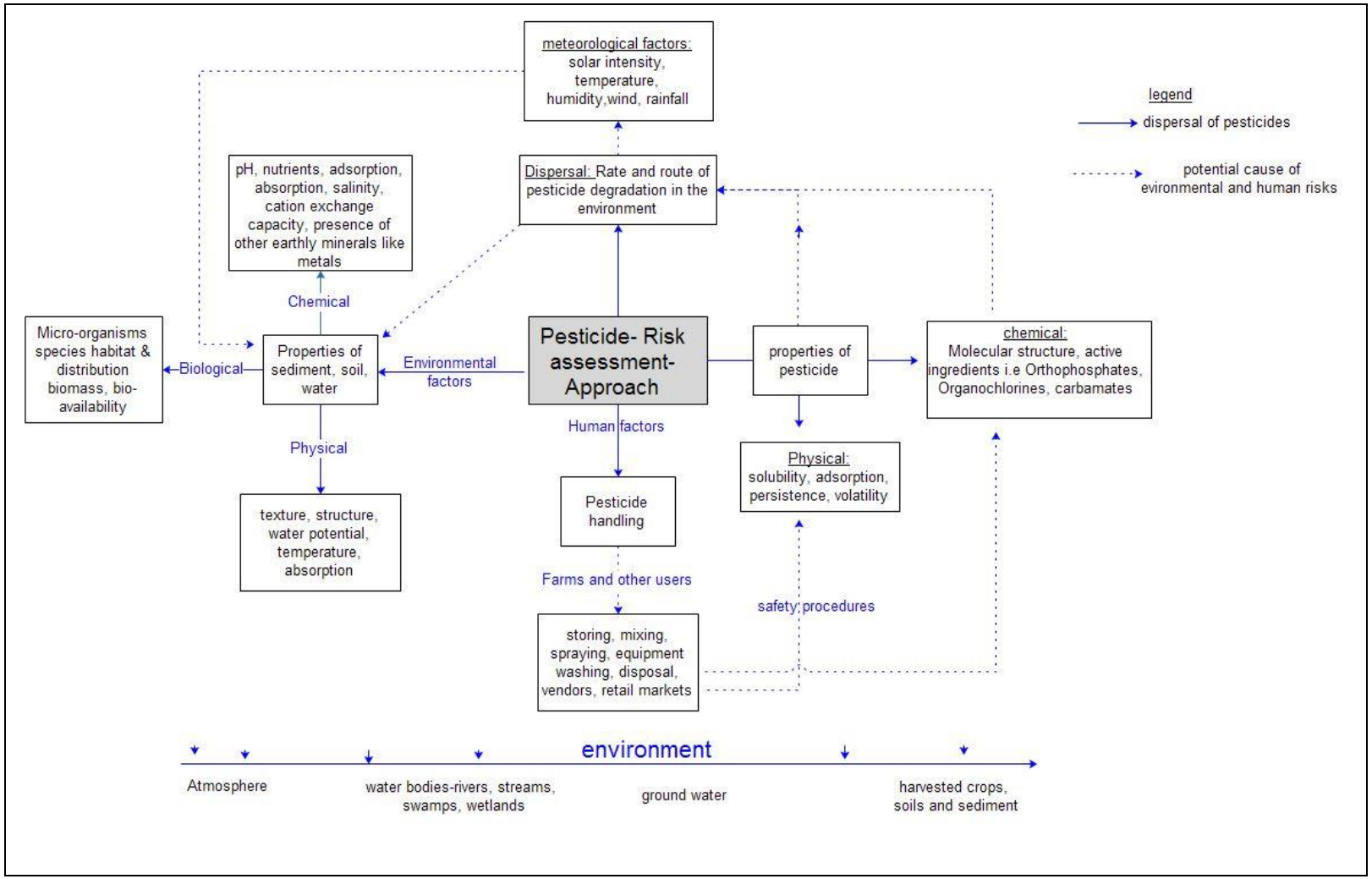


Figure 4.7: An outline of the parameters to be considered when conducting human and environmental risk assessment of pesticides (extracted from Mackay et al. (1997).

4.4.1 Pesticide handling in Tanzania

In Tanzania, pesticide formulations and distribution are widely achieved by licensed pesticide retailers (Lekei *et al*., 2007). Small scale farmers purchase small quantities of pesticides from easily accessible pesticide retailers in the agricultural areas, whereas large scale farmers procure pesticide directly from the licensed pesticide distributors or formulators who import both active and inert ingredients from overseas (Lekei *et al*., 2007). Formulators provide information or pesticide data on the labels that might include restrictions for use under certain field application conditions and other necessary details such as handling, antidote in case of human poisoning, and other necessary information or toxicity to non-target organisms.

Nevertheless, trade liberalization in Tanzania, high poverty levels, farmers' limited knowledge, negligence and lack of monitoring or regulations on pesticide emissions to environment, envisage an increased potential risk to human and the ecosystem at large. A few studies have documented various scenarios of unsafe handling of pesticides by farmers in Tanzania that pose potential human and environmental risks (Lekei *et al*., 2014; Ngowi *et al*., 2001; Stadlinger *et al*., 2011). During preliminary field visit and interviews with the plantations managers, it was revealed that pesticides distribution to three plantations is done in large volumes and in a contractual manner, while in the rest of five surveyed plantations pesticides are purchased from retail shops or supplied by pesticide vendors directly to their farms in the amount and on the time needed. These small scale purchases and distributions might be associated with unsafe handling during repackaging into small volume containers which are dumped in the fields after use, a situation which was witnessed during sampling campaign and reported elsewhere in Tanzania (Lekei *et al*., 2004; 2014). Small scale farmers are tempted to rely on the instructions provided on the pesticide labels, which sometimes is in foreign language (English), causing difficulties in appropriate pesticide handling. At times farmers rely solely on the instructions given by the pesticide retailers/vendors who are usually contented to promote the sale of their products in stock. Negligence, which is accelerated by lack of knowledge and awareness on chemical safety issues, enhances the mishandling of pesticides in Tanzania. However, it was revealed that all eight plantations listed in this study, had either a well-trained and experienced agronomist or agricultural extension officer, who, apart from other farm duties, was entrusted, with providing training to farm workers on issues of occupational health and safety. It was also informed that farm agronomists and extension officers were responsible for assuring safe storage of agrochemicals and supervises pesticide apportioning when necessary. Two

plantations had an occupational health and safety department, which ensured the use of personal protective equipment and all issues related to workers safety in their plantations. This is a positive indication of reducing human exposure to toxic chemicals. Besides, the same two plantations claimed to return empty pesticide containers to the suppliers/distributors for re-use.

Furthermore, the Tanzania Pesticide Research Institute provides a list of legally registered pesticides as explained in details in Section 1.3.6, which are licensed for use depending on crop and targeted pest. However, what might be lacking is monitoring and evaluation programs on whether the categorical use of such pesticides in the farms adheres the requested permit, in order to safeguard the ecosystem. Another constraint is that small scale farmers might not be aware of the provisions of regulatory boards, so they tend to trust the information provided by the pesticide distributors, which might be insufficient or misleading depending on the knowledge of the provider. Some of the factors that might accelerate the risk of pesticide usage in the Kilombero Valley Ramsar Site and in other wetlands of Tanzania are: lack of national legal framework on chemical management and safety issues that would give guidance on disposal of obsolete pesticides or empty containers; insufficient data on the biological and ecological impacts of pesticides to aquatic ecosystems; and lack of sanctions related to deliberate or negligence of contamination of aquatic ecosystem due to pesticides emissions. In Tanzania, as in many parts of sub-Saharan Africa, wetlands are used for agriculture and are potential sources of freshwater for domestic use. Therefore, contamination of wetlands through agrochemicals poses a great risk to human health. Furthermore, the same study by Lekei *et al*. (2007) revealed that there were no laboratories for quality control and assurance of the finished pesticide products in all seven pesticide formulation plants that were inspected in Tanzania. Failure to conduct quality control, research and development by such plants indicates that pesticide with impurities or which are substandard might be distributed in the market. Consequently, such factors might contribute to improper disposal of old brands or might be a reason for farmers to mix or change pesticide brands as responded during questionnaire survey in Section 3.8.3. Therefore, lack of quality control laboratories, emission standards, and monitoring pesticide usage by Tanzanian national authorities signposts the unforeseen risk of the current policy of modernization and privatizing agricultural activities in the southern highlands of the country where sensitive ecosystems, like Kilombero Valley Ramsar Site and wildlife reserves are located.

4.4.2 Current Tanzanian government agricultural policies: Pesticides-ecological risks

In the current Tanzanian government policy to reduce mass poverty and increase food security through modernization of agriculture and agribusiness investments, massive use of pesticides and other agrochemicals will not be circumvented. Kilombero valley is one of the target areas for rice and sugarcane production due to its fertile soils, adequate water for irrigation and inundated areas. These agribusiness investments under SAGCOT project, is earmarked in the southern highlands of Tanzania, which contains sensitive ecosystems such as Ramsar wetlands, mountains, rivers, national parks and wildlife corridor. Different types/classes of pesticides residuals were detected in the sediment-soil-water samples of Kilombero valley (Section 3.1.4). Such pesticides have the potential to pose risks to non-target aquatic species. For instance, aquatic plants are susceptible to toxicities of most of organophosphates when they are released in sufficient amount into environment (Battaglin & Fairchild, 2002). However, toxicities depend on the concentration of the pesticides, physicochemical properties of active ingredients, or its metabolites and species sensitivity to the toxicant (Cáceres *et al*., 2008). Surface runoff, during high rainfall seasons, might contain pesticides in sufficient quantities to results into potential toxic effect to non-target aquatic organisms in the Kilombero wetland ecosystem. Richards and Baker (1993), reported that average concentration of pesticides in the agricultural watersheds were highly correlated with the amount of pesticides applied, mode of applications and chemical properties of the pesticides, while, peak concentration in the studied streams were inversely related to size and flow of the streams. During rainfall seasons, surface runoff that follows pesticides application might result into peak discharge of agrochemicals and thus concentrations in the small streams might rise rapidly and decline slowly thereafter. Therefore, for Kilombero flood plain, the elevated pesticides concentrations in the small streams might be observed during February to May rainy season as shown earlier in Fig. 3.40 and low concentrations the rest of the year. However, in this study although in low concentrations, pesticides were detected more frequently in water samples collected during rainy season, March 2012, than those collected during dry season. Sediment samples had an increased frequency of pesticide detection during dry season, January 2013 sampling. The increased detection of pesticides in water samples during rainy season, is attributed by dilution and re-suspension of contaminants during rainy seasons, while in dry seasons, contaminants or pesticides are bound to sediments or soil particles, increasing their potential to be detected in dry season sediment and soil samples. High degradation potential and dissipation of pesticides in the tropical climates, under elevated temperatures, humidity, strong wind movement, high precipitation rates and soil moisture as

shown in the monthly meteorological data for Kilombero sugarcane and rice plantations, increase the susceptibility of aquatic organisms to chemical toxicity in the Kilombero Valley.

4.4.3 Agronomic activities in the Ramsar site: a quest for sustainability

Over the last two decades the use of Kilombero wetlands for agriculture has increased because of increasing population of both pastoral and agro-pastoral communities for livelihood activities (TAWIRI., 2008; 2009; Mombo *et al*., 2011). Both large scale and small-scale sugarcane and rice paddy agriculture is practiced within the valley. Farm-weed management is a great challenge for farming activities and thus weed control using herbicides is prominently opted by farmers and other big plantations for rice and sugarcane farming. The Ramsar Convention of 1971 requires the contracting parties to create their national wetland policies to suit their local environment (Ramsar, 1971). The Convention further promotes the wise use and management of Ramsar wetlands through local communities' participation (RCB, 1993). Nevertheless, insufficient management plans for this Ramsar wetlands, has led to intensive and continuous use of pesticides and other agro-chemicals within the valley. Therefore, long term environmental risk and destruction of ecological integrity of the Kilombero Ramsar wetlands is anticipated

CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

Eco-toxicity tests criteria are measured as impact of stressors on biochemical, physiological or metabolic functions of test organisms. The increasing use and acceptance of biotests for risk assessment has led to the development of standardized test methods that are universally applied in order to assure reliability of test methods and reproducibility of results. However, such biotest methods have been largely established in temperate countries using temperate organisms with test criteria that suit the test species´ habitat. The major challenge that remains is the universal application of the test organisms and methods developed for temperate region to tropical climates where the fate and behavior of toxicants in the environment is affected differently by climatic conditions (Daam & Van den Brink, 2010; Laabs *et al*., 2002a). Moreover, tropical species are capable of adapting to extreme weather conditions and thus develop different physiological adaptation and sensitivity to certain toxicant, therefore using a laboratory temperate model organisms might under or over-estimate the toxicity of such toxicants for the tropical field exposure (Lahr, 1997). Therefore, this study was conducted in order to: (i) apply three standardized bioassays for temperate regions to determine eco-toxicity of water, soil and sediment samples of freshwater tropical wetlands; (ii) critically discuss the suitability of temperate biotest batteries for assessing pesticide contamination in tropical agronomic systems; and (iii) estimate potential risks to humans and aquatic life based on chemical analysis data of soil-sediment-water samples from agricultural fields. The study area was Kilombero Valley Ramsar Site, Tanzania. Fig. 4.8 summarizes the potential strengths, weaknesses, opportunities and threats (SWOT) of conducting eco-toxicological studies in Tanzania. The following conclusive remarks can be derived.

- The study has shown that *V. fischeri* and *A. globiformis* bioassays can be used in tropical climates and to identify contaminated hotspots. However, the chronic *P. subcapitata* bioassay could not provide enough information on the toxicity of sample matrices. The results of *P. subcapitata* obtained in this study with Kilombero sediment-water-soil samples shows that there might be a limitation of the suitability of using *P. subcapitata* bioassay for eco-toxicological assessments of nutrient rich, agricultural soils or sediments samples. Thus, there might be a possibility of underestimating the toxicity of samples matrices collected from agricultural fields, especially for sediment and soil samples that are rich in nutrients

and are contaminated with low concentration of pesticides. Consequently, it is recommended to incorporate the measurement of delayed and prompt light emitted by photosynthetically active cells. If both chronic and acute *P. subcapitata* bioassays are applied, supplementary toxic information of herbicides can be derived from the environmental samples.

- About prompt and delayed fluorescence measurement, it was shown that it is possible to predict, experimentally, the presence of herbicides that inhibits PS II and pesticides with different mode of action in the samples collected from agricultural fields. This study has demonstrated a different approach of estimating the toxicity of certain chemicals using acute conditions or a short-exposure period based on the prompt fluorescence (PF) and long delayed fluorescence (DF) on a timescale of 80–100 microseconds. Using both PF and DF in acute tests, may be a useful tool to assess the presence of photosystem II inhibitors based on rapidly estimating the inhibitory or stimulatory effects that the chemicals would have had on the conventional 72 hours algal growth inhibition tests. The method used in this study is not only simple and rapid, but consumes less time and labor. Therefore, a further critical assessment of this method on array of environmental samples for risk assessment is recommended, because a few samples used in this study could not yield adequate information. Relatively new methods that measure direct responses of pesticides to test organisms are available. Such methods use a Pulse Amplitude Modulated (PAM) fluorescence-based methods to assess the effects of photosystem II inhibitors (herbicides) (Dorigo & Leboulanger, 2001) or single and mixture effects of pesticides and their degradation products (Kim Tiam *et al*., 2014). In this study, one multimode fluorescence reader could be used for all tests that were applied without requiring an additional and expensive apparatus such as PAM, which was not available in our laboratory by the time this study was undertaken.
- Eco-toxicological assessments indicate the total of all bioavailable toxic substances to the test organisms, while chemical analysis of environmental samples are limited to the subjective selection based on the history of the area, or contaminants of interests. Since not all contaminants may be known and metabolites formed during biodegradation process might not be taken into consideration during analytical assessment, eco-toxicological tests provide valuable additional information for early screening purposes.

- Another yield from this study is that another microbial test using yeast, *S. cerevisiae* can be adopted for use in assessing toxicity of contaminants in the terrestrial ecosystem. If this test is well established and standardized, it can be used under a classical laboratory setting. The unicellular yeast, *S. cerevisiae*, which is widely distributed, can be used to assess environmental impacts of fungicides. Besides, *S. cerevisiae* is non-pathogenic, fast growing in both simple and cheap medium, and easy-to-manipulate. The experimental design and laboratory procedures described in this study, provide potential information for further development of *S. cerevisiae* bioassays, especially on assessing the exposure to organic fungicides such as prochloraz and thiabendazole, which could not show toxic responses in this study. Moreover, resazurin is non-toxic to cells and users, thus it does not require special handling or disposal methods. Higher sensitivity is anticipated in fluorescence detection that has the flexibility of using two wavelength options, the excitation and the emission wavelengths, to measure internal reference signals.
- Furthermore, important information on the environmental and human risk of agrochemicals use in Kilombero Ramsar site can be attained from this study. The continuous use of pesticides in these wetlands poses a great risk to human health, wildlife and aquatic organisms that can be exposed to interminable low concentration of both active and inactive ingredients of the pesticides, and their metabolites. Inert ingredients of pesticides are chemically inactive to the formulated product but they may be biologically or chemically active to other chemicals or to the ecosystem in general. The use of phosphate fertilizers and metal containing pesticides increase the risk of heavy metals and radionuclides contamination in the wetland and the whole river basin.

In summary, the information reported in this study promotes the potential of using bioassays for setting water quality criteria to protect the freshwater ecosystem of Kilombero Valley Ramsar site and other wetlands that are faced by human pressure from agricultural activities in Tanzania. This is the first study that tries to analyze toxicity of Tanzanian sediment, soil and water using different test organisms. Analytical parameters such as grain size, dry weight, salinity, C/N ratio, are documented. Furthermore, the screening of different pesticides in the samples, heavy and trace metals also provides an insight on the status of different samples collected from areas surrounding rice and sugarcane plantations of Kilombero valley. A potential risk of the current agronomic activities in the Kilombero Valley Ramsar site is established by combining biological, chemical data and field observation results. The results of this study provide broad risk information to multi-

stakeholders and the results can be used to raise awareness or recommend an initiative management plan for sustainable wetland agricultural practices. Ecotoxicology using simple, fast and cost effective bioassays in a classical laboratory might help to provide strategies for assessing the toxicity of chemicals in the Tanzanian ecosystems.

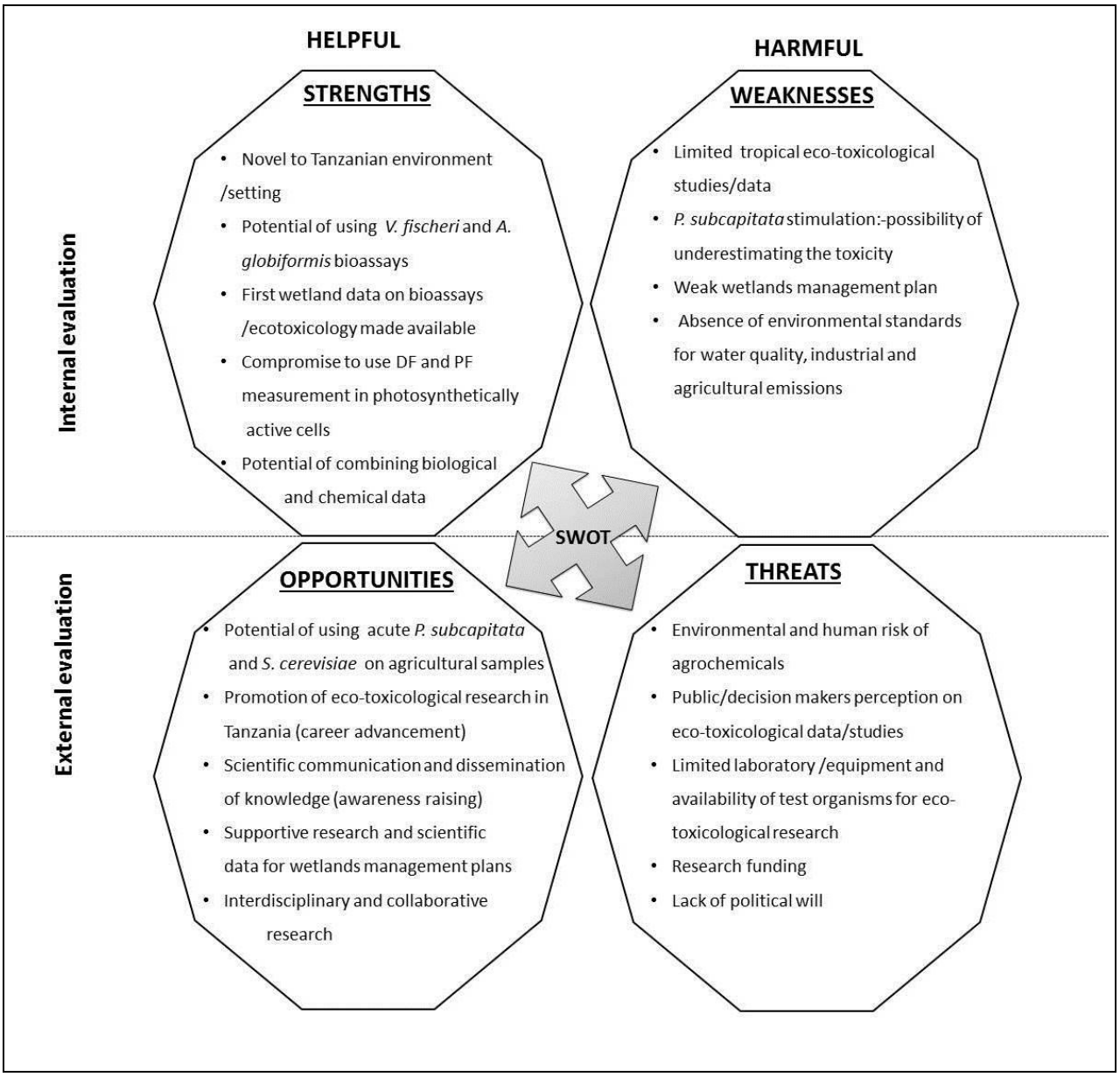


Figure 4.8: SWOT-diagram (strengths, weaknesses, opportunities and threats): Internal and external evaluation of the suitability and possibility of using bioassays in Tanzania.

5.2 Future research

Published pesticide data from Tanzania typically relate to quantification of pesticide residue in precise environmental matrices. Integrated studies relating exposure and effects are limited. In order to assess the environmental impact of pollutants, there is a necessity to consider both

exposure information and ecosystem effect. The restricted and limited scope of eco-toxicological studies of impacts of toxic chemicals such as pesticides in Tanzania and other tropical African countries in general point out the area of research priority by eco-toxicologists. Identification of sensitive organisms of ecological value that can be used in toxicity testing is of vital importance.

There is insufficient eco-toxicological data on impact of toxic chemicals emitted by agribusiness investments and manufacturing industries in Tanzania. The lack of scientific data hinders the capacity to manage decisions by policy makers and thus political decisions might over-rule scientific reality. Research is needed in Tanzania to develop a better insight into the potential impacts of repetitive low-level exposure of toxicants to aquatic organisms. Pesticides such as diuron, desmethydiuron, glyphosate, chlorpyrifos, propoxur, atrazine, and hydroxyl-atrazine, which were detected in the water and sediment samples of Kilombero valley ecosystems, can be regarded as a priority of future research in monitoring rice and sugarcane production in the wetlands ecosystems.

There is limited data on agricultural safety or environmentally accepted standards of heavy metal or radioactive elements concentrations (impurities) in the *Minjingu* phosphate fertilizers that is locally mined in Tanzania and quite often used by farmers. It is therefore recommended to conduct further research on the extent of use of *Minjingu* and other phosphate fertilizers in the Kilombero valley by farmers, and its associated risk of heavy metals and radionuclides contamination in soils and food crops such rice, maize, cereals and sugarcane.

In order to develop long-term sustainable national standards on water quality by Tanzanian Bureau of Standards (TBS), future research depending on the perspective of acceptable risks, standardized or relevant methods and model evaluation, which requires expertise from ecology, toxicology and chemistry is recommended. The concept of acceptable risk levels should be assessed in relation to well-established standards and guidelines, such as OECD, European unions or any other renowned standards.

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APPENDICES

Appendix 1: Questionnaire on pesticide use in the Kilombero Valley Ramsar Site

Dear agricultural farm managers and agronomists of Kilombero Plantations Co.Ltd, Kilombero Valley Teak Company, Mbingu sisters, Idete agricultural prisons, Kiberege agricultural prison, Tanzania Agricultural Centre and Kilombero Sugarcane Company. I am currently conducting my PhD research study (2012/14) under Sokoine University of Agriculture, Tanzania, in collaboration with Applied Aquatic Toxicology Research Group of Hamburg University of Applied Sciences, Germany. The purpose of the study is to investigate the potential effects of pesticides on the environmental and ecological integrity of the Kilombero Valley Ramsar site. The first phase of this survey will base on the large scale farmers and later the study will be extended to small scale farmers.

Following my previous visit in your office/plantations, and as per our conversation on pesticide use, I would request you to fill up this questionnaire which investigates the trend and type of pesticide used in the sugarcane, rice and teak plantations for the past one decade. It is for research /academic purposes only. Please answer the questions to the best of your knowledge. Answers will be kept completely confidential and will only be presented in a summary format. For good records I have deduced some of the questions to be answered in a table format to guide you on the important sections to fill in.

General information:

Company/plantation name	
Physical address and telephone	
Contact person	
Position	
Contact person E-mail address:	
What is your profession and highest level of education?	
What is the total size of the land owned by the company?	
What is the approximate area (of the total land) that is used for cultivation?	

Would you like to get the results of this pesticide toxicity survey for the Kilombero Valley Ramsar site ecosystem? *Please tick (v)* YES ( ) NO ( )

Continue with the survey.

1. List all type of the pesticides/herbicides that have been used in the plantation for the past 5-10 years. If the *product name is unknown but active ingredient is known, enter active ingredient in the column provided.*

Product/ Trade name	*Chemical name/active ingredient*	*used for /used as:*	*When applied, which season of the crop cycle*	*Manufacturer*	*Agent/Distributor*
Example: Hansunil 600EC	Propanil 200g/L, Thiobencarb 400 g/L	Herbicide: control of grass, sedges and broad leaf weeds in rice farms	Post emergence herbicide	Hangzhou Agrochemicals Industries Co. LTD, China	Hangzhou Agrochemicals (T)ltd : P.O Box 16368-Arusha - Tanzania

Any comments:

---

2. List of the pesticides/herbicides used in the plantation for the past 10-15 years. If the *product name is unknown but active ingredient is known, enter active ingredient in the column provided.*

Product/ Trade name	*Chemical name/ active ingredient*	*used for /used as:*	*When applied, which season of the crop cycle*	*Manufacturer*	*Agent/ Distributor*

Any comments:

---

3. List all type of pesticides/herbicides used in the plantation as for this year up to the past 5 yrs (0-5 years). If the *product name is unknown but active ingredient is known, enter active ingredient in the column provided. If the chemical has also been used continuously over years you can also list it here.*

Product/Trade name	*Chemical name/active ingredient*	*used for /used as:*	*When applied, which season of the crop cycle*	*Manufacturer*	*Agent/ Distributor*

Any comments:

---

4. List of the current pesticides, which are under trial/experimentation for further applications in the plantations to improve yield. If the *product name is unknown but active ingredient is known, enter active ingredient in the column provided.*

Product/Trade name	*Chemical name/active ingredient*	*used for /used as:*	*When applied, which season of the crop cycle*	*Manufacturer*	*Agent/ Distributor*

Any comments:

---

5. What is the major mode of transport in delivering the product to your premises? *Please insert numbers 1-4 in terms of priorities.*

Air transport ( ) Rail transport( ) Road transport (auto -vehicles, trucks, motorcycles ( ) Water transport, ship, boat etc. ( )

Other (*specify*) ……

6. Approximately what percentage of product delivered to your premises arrives damaged/ unsealed or broken? _____%

7. Can you estimate in the table below, the most frequently used pesticides in percentage - according to the **quantity of product used** in your plantations/farms?

	Insecticide	**Herbicide**	**Fungicide**	**Nematicide**	**Rodenticide**	**Others…(***please name)*
First						
Second						
Third						
Fourth						
Fifth						
sixth						

8. What is the rate of application of the above named pesticides in the crop product production cycle? *Please estimate the amount of chemical in (kg or tones or litres-etc per hectare) from farm preparation to harvest.*

Chemical	**Insecticide**	**Herbicide**	**Fungicide**	**Nematicide**	**Rodenticide**	**Others: *specify***
Amount per hectare						

9. Do you always buy the same brands of pesticides used in your plantation, or do you regularly
change brands of pesticides? *Please tick(v)*
1. ( ) Always the same. 2. ( ) Change regularly. 3. ( ) Change sometimes

If change, answer Question 9.1

9.1 What is the reason why you regularly change brands of pesticides? *Please tick (v)*
1. ( ) The new brand of other pesticide companies is better
2. ( ) The old brand is no more effective to pests
3. ( ) Following recommendation from retailers/ pesticides companies
4. ( ) Following recommendation from agricultural officials
5. ( ) Others (*please specify*) _____

10. When you buy pesticides, does it happen sometimes that the container(s) has no label? *Please tick (√)*
1. ( ) Never happen 2. ( ) It does happen sometimes 3. ( ) Often

11. What types of equipment are used during pesticide application? (*Please tick v)*

i	Ground sprayer ( )	viii	Spray line, hand-held ( )
ii	Trigger pump/compressed air ( )	ix	Manual placement ( )
iii	Aerosol generator/fogger ( )	x	spot application ( )
iv	Hand-held granular/dust application ( )	xi	Sprayer- Backpack ( )
v	Aerial application equipment ( )	xii	via irrigation ( )
vi	Soil injector ( )	xiii	hooded sprayer ( )
vii	High pressure fumigator ( )	xiv	Boom ( )
xv	Others *(please specify)* ……		

12. Do you mix different brands of pesticides before application?
( ) Yes ( ) No

12.1 If YES, do you mix the required (by the label) quantity of each brand in the same water?
( )Yes ( ) No

12.2 If YES, please specify the brand and mixture you normally use.

Water volume of spraying container (in Litres)	Brand Name of the chemicals mixed	Amount you mix (i.e. grams or mL) per spraying container

12.3 What is the main reason for mixing the pesticides this way? *Please tick (√)*

( ) Unsure about the quality of pesticides
( ) Uncertain about the effectiveness of pesticides for a particular pest
( ) Imitating other applicators
( ) Following the suggestion of others
Other reason (please specify) ……

13 On a scale of 1-5, how much risk do you think your workers are exposed to while applying pesticides to the field/farms? *Please write the number on the brackets provided according to your knowledge.*

( ) No risk at all
( ) Some small risks
( ) A medium amount of risk
( ) A large and significant amount of risk
( ) Dangerous and very toxic risks
( ) I do not know

14 What are the names of the nearest rivers/river tributaries that receive effluents from your farms and from the neighboring village?

N*ame* and *location of the plot*	*size* of the plot	Neighboring rivers (*specify-in west, east, south or northern)*	Estimated distance (in *meters or km*) from the plot/farm to the river

15 Do you use chemical fertilizers in your plantations? *Please tick* (√)

YES ( ) NO ( )

15.1 Which types of fertilizers do you use? *Please name*

i……ii……
iii……iv……
others……

15.2 How are the fertilizers applied?

……
……
……

15.3 Do you mix fertilizers and pesticides during applications? *Please tick* (v)

( ) YES, but sometimes ( ) NOT AT ALL ( ) YES, always

15.4 If YES, which types of pesticides are mixed with fertilizers? And in what ratio?

Name of fertilizers and mixing proportions
i.
ii.
iii.
iv.
v.

Thank you very much for your participation in this survey. Your answers will be extremely useful for my research. Again, I assure you that all the answers you have provided in this survey will be kept strictly confidential and will never be revealed to any other person outside our research group.

Sincerely

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Appendix 2: DIN- Medium :Concentration of nutrients in the test solution for Algae, *P. subcapitata,* Growth Inhibition Test (DIN-EN ISO 8692, 2004)

Stock solution **(DIN- medium)**	Nutrients	Mass concentration in the stock solution	Final mass concentration in the test mixture
Stock solution 1: **DIN-1** (Macronutrients)	NH4Cl	1.5 g/L	15 mg/L
	MgCl2.6H2O	1.2 g/L	12 mg/L
	CaCl2.2H2O	1.8 g/L	18mg/L
	MgSO4.7H2O	1.5 g/L	15mg/L
	KH2PO4	0.16g/L	1.6mg/L
Stock solution 2: **DIN-2** (Fe-EDTA)	$FeCl_3.6H_2O$	64 mg/L	64 µg/L
	$Na_2EDTA.2H_20$	100 mg/L	100 µg/L
Stock solution 3: **DIN-3** (trace elements)	H_3BO_3 [a]	185 mg/L	185 µg/L
	$MnCl_2.4H_2O$	415 mg/L	415 µg/L
	$ZnCl_2$	3.0 mg/L	3.0 µg/L
	$CoCl_2.6H_2O$	1.5 mg/L	1.5 µg/L
	$CuCl_2.2H_2O$	0.01 mg/L	0.01 µg/L
	$Na_2MoO_4.2H_2O$	7.0 mg/L	7.0 µg/L
Stock solution 4: **DIN-4** ($NaHCO_3$)	$NaHCO_3$	50 g/L	50 mg/L
[a] H_3BO_3 Can be dissolved by adding 1mol/L NaOH.			

Appendix 3: 1/3 DSM medium for *A. globiformis* pre-culture

Component	Amount
Casein peptone, tryptic digest	10.0 g
Yeast extract	5.0 g
Glucose	5.0 g
Agar	15.0 g
NaCl	5.0 g
Total volume (distilled water)	1000.0 mL
Adjust pH to 7.2 - 7.4	

Appendix 4: ASW-Artificial Seawater for *V. fischeri* bioassay

Mineral Salts	**Concentration** (mM)	**g/L**
NaCl	250	14.61
$CaCl_2$-$2H_2O$	10	1.4702
KCl	10	0.7455
$MgCl_2$-$6H_2O$	50	10.165
Add the salts to distilled/Deionized Water mix and bring to volume		
Adjust pH 7.2 ± 0.2 (with 0.1 M NaOH)		
then autoclave		

Appendix 5: The fuzzy empirical rules for classifying *P. subcapitata* (AGI), *A. globiformis* (BCA) and *V. fischeri* (LBT) bioassays endpoints inhibition

		Condition of variable attributes (toxic response)							**Total Weight**
Rule		**AGI**		**LBT**		**BCA**		**Classification**	
1.	*If*	Low or no toxic response	*and*	Little or no toxic response	*And*	Little or no toxic response	*Then*	Low or no potential risk	1
2.	*If*	Low or no toxic response	*and*	Moderate toxic response	*And*	Moderate toxic response	*Then*	Critical risk	1
3.	*If*	Low or no toxic response	*and*	Little or no toxic response	*And*	Highly toxic	*Then*	Low or no potential risk	1
4.	*If*	Little or no toxic response	*and*	Moderate toxic response	*And*	High toxic responses	*Then*	Low or no potential risk	1
5.	*If*	High toxic response	*and*	Moderate toxic response	*And*	High toxic response	*Then*	Elevated critical risk	1
6	*If*	Moderate toxic responses	*and*	Little or no toxic response	*And*	High toxic response	*Then*	Critical risk	1
7.	*If*	Moderate toxic response	*and*	Moderate toxic response	*And*	High toxic response	*Then*	Critical risk	1
8.	*If*	High toxic response	*and*	High toxic response	*And*	High toxic response	*Then*	Elevated critical risk	1
9.	*If*	High toxic response	*and*	High toxic response	*And*	Moderate toxic response	*Then*	Elevated critical risk	1
10.	*If*	Moderate toxic response	*and*	High toxic response	*And*	Little or no toxic response	*Then*	Critical risk	1
11.	*If*	High toxic response	*and*	Little or no toxic response	*And*	High toxic response	*Then*	Elevated critical risk	1

12.	*If*	Moderate toxic response	*and*	Moderate toxic response	*And*	Moderate toxic response	*Then*	Critical risk	1
13.	*If*	Low or no toxic response	*and*	High toxic response	*And*	Moderate toxic response	*Then*	Critical risk	1
14.	*If*	High toxic response	*and*	Moderate toxic response	*And*	Little or no toxic response	*Then*	Critical risk	1

Appendix 5 cont.

15.	*If*	Low or no toxic response	*and*	Moderate toxic response	*And*	Little or no toxic response	*Then*	Low or no potential risk	1
16.	*If*	High toxic response	*and*	Moderate toxic response	*And*	Little or no toxic response	*Then*	Critical risk	1
17.	*If*	High toxic response	*and*	Little or no toxic response	*And*	High toxic response	*Then*	Elevated critical risk	1
18.	*If*	Little or no toxic response	*and*	High toxic response	*And*	Little or no toxic response	*Then*	Low or no potential risk	1
19.	*If*	Moderate toxic response	*and*	Moderate toxic response	*And*	Little or no toxic response	*Then*	Critical risk	1
20.	*If*	High toxic response	*and*	High toxic response	*And*	Little or no toxic	*Then*	Critical risk	1
21.	*If*	Moderate toxic response	*and*	High toxic response	*And*	Moderate toxic response	*Then*	Elevated critical risk	1
22.	*If*	High toxic response	*and*	Little or no toxic response	*And*	Little or no toxic response	*Then*	Low or no potential risk	1
23	*If*	Little or no toxic responses	*and*	Little or no toxic responses	*And*	Moderate toxic responses	*Then*	Low or no potential risk	1
24.	*If*	Little or no toxic	*and*	High toxic responses	*And*	High toxic responses	*Then*	Elevated potential risk	1
25.	*If*	High toxic responses	*and*	Little or no toxic responses	*And*	Moderate toxic responses	*Then*	Critical risk	1
26.	*If*	Little or no toxic responses	*and*	Moderate toxic responses	*And*	Little or no toxic responses	*Then*	Low or no potential risk	1
27.	*If*	Moderate toxic responses	*and*	Little or no toxic responses	*And*	Little or no toxic responses	*Then*	Low or no potential risk	1

Total weight for responses in three bioassays=permitted range of certainty factors

Appendix 6: List of registered pesticides for use in rice and sugarcane in Tanzania (TPRI, 2011)

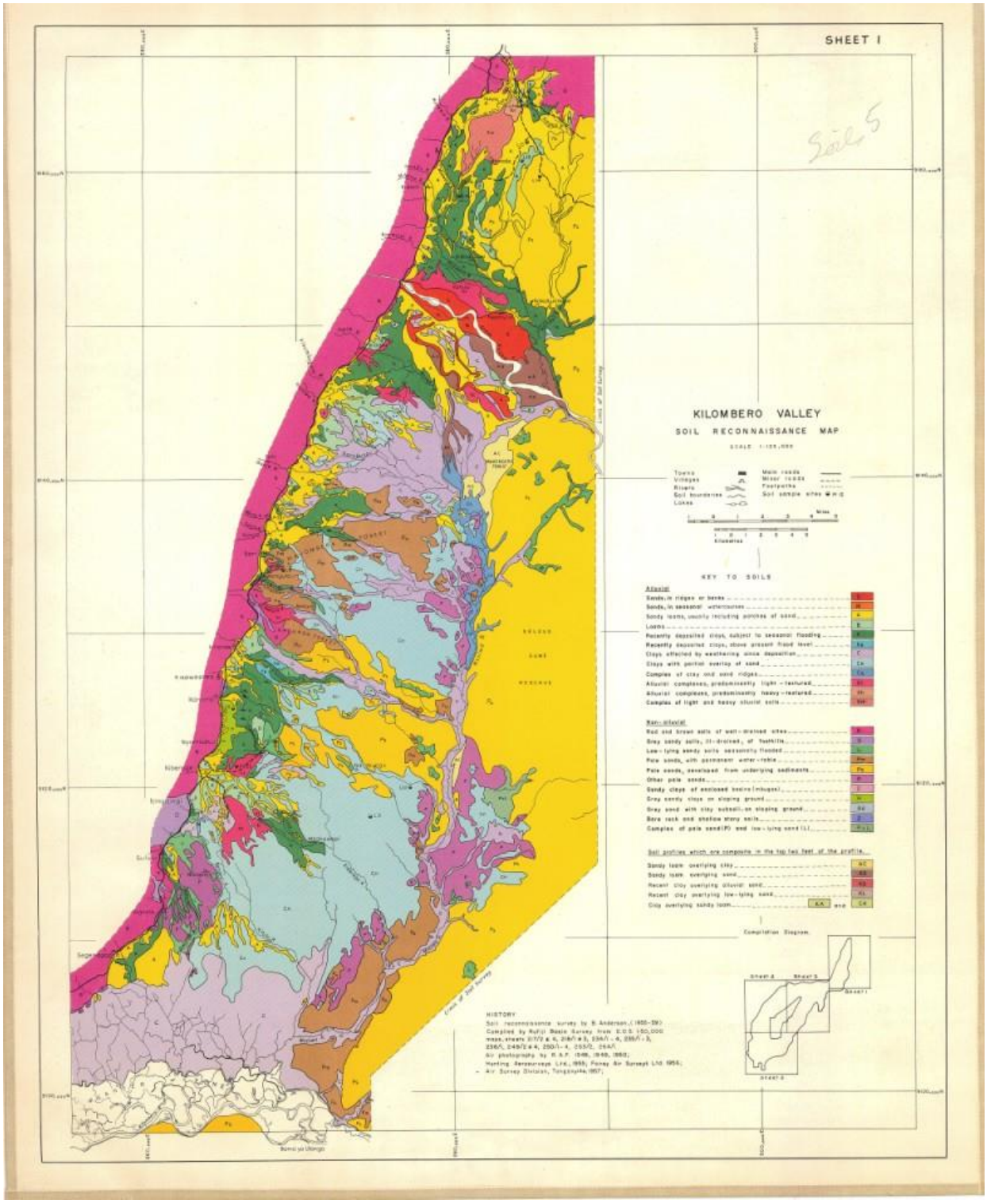
(Tropical Pesticides Research Institute (TPRI), Made under section 18 of the Plant Protection Act, 1997 and Plant Protection Regulations GN 401 of 1999)

1. FULL REGISTRATION CATEGORY		
Trade name	Common Name	Usage
1A: HERBICIDES		
2,4-D Amemine 720SL	2,4-D Amine 720g/l	Weeds control under minimum tillage in rice
Albatros 500SC	Ametryn + Atrazine	Pre-emergency weeds control in sugarcane
Alligator 500EC	Pendimethalin 500g/l	
Anaconda 500SC	Ametryn 500g/l	
Atramet Combi 50SC	Atrazine 235g/L + Ametryn 24	Grasses and broad leaf on weeds sugarcane and horticultural crops
Atranex 50 SC	Atrazine 500g/L 5g/L	Weeds control on sugarcane plantations
Atraweed 50SC	Pendimethalin 500g/l	Control of weeds in Sugarcane
Baiuron 800SC	Diuron 800g/l	Control of broad leaf and grass weeds in Sugarcane
Bajuta MSMA 720SL	MSMA 720g/L	
Banstar 250EC	Oxidiazon 250g/L	Weed control in transplanted lowland rice
Baphosate 360SL	Glyphosate 360g/L	Post – emergence weeds control in rice and sugarcane
Bispicice 400 SC	Bispiribac – sodium 400 g/l	Controlling weeds in irrigated rice
Bencor 480SC	Metribuzin 480g/L	Controlling weeds in maize
Brigadier 750WG	Halosulfuron	Selective weeds control in sugarcane.
Buccaneer 960EC	Metolachlor	Pre-emergency weeds control in sugarcane
Dash 750WG	Hexazinone 750g/Kg	Weed control in sugarcane
Delphi 720SL	2,4-D Amine 720g/l	Selective post emergence weeds control in sugar cane
Diablo 800SC	Diuron 800g/l	Pre-emergency weeds control in sugarcane
Diablo 800WG	Diuron 800g/l	
Dual Gold 960EC	S-Metolaclor-960g/L	Protect maize, beans and sugarcane against pre-emergence weeds
Fusilade Forte 150EC	Fluazifopbutyl 50g/L	Used as a ripener in sugarcane
Galigan 240EC	Oxyfluorfen 240g/L	Weeds control on sugarcane plantations
Hansunil 600EC	Propanil + Thiobencarb	general weed control in lowland rice
Hexarone 600SL	Diuron468g/l + Hexazinone 132g/L	weed control in sugarcane
Jaguar 900EC	Acetochlor 900g/L	Pre-emergency weeds control in sugarcane
Kalif 480EC	Clomazone 480g/L	Weed management in lowland rice under irrigation conditions.
Krismat 75 WG	Trifloxysulfuron Sodium 18.5g/Kg + Ametryn 731.5g/Kg	Control of cyperus, dicot and some grasses in sugarcane
Lumax 537.5SE	Terbuthylazine 125g/L + Mesotrione 37.5g/L	Weeds control in maize and sugarcane
Mobuzine 480SC	Metribuzine 480g/L	Weeds control in sugarcane
Papyrus 100WP	Pyrazosulfuron 100g/KG	General weed control in lowland rice
Pencal 500EC	Pendimethalin 500g/L	Weeds control on sugarcane plantations
Pendi 500 EC	Pendimethalin 500g/L	Control of weeds in sugarcane (*Saccharum spp*.) plantation
Piranha 360SL	Glyphosate	Non-selective weeds control in sugarcane
Piranha 360WG	Glyphosate	
Rebel 500WG	Chlorimuron	Selective weeds control in sugarcane.
RiceBack 400 SC	Bispiribac – sodium 400 g/l	Controlling weeds in irrigated rice
Rilor 500EC	Pretilachlor 500g/L	Grass and broadleaf weeds control in transplanted rice.
Ripanil 360EC	Propanil 360g/L	
Ronstar 250EC	Oxidiazon 250g/L	Weeds control on rice, flower and horticultural crops
Sabre 720SL	MSMA	Selective post emergence weeds control in sugar cane
Satunil 60EC	Benthiocarb + Propanil	Rice against gramineous cyperaceous weeds.
Saturn 50 EC	Benthiocarb 500g/l	
Sencal 480SC	Metribuzin 480g/L	Weeds control on sugarcane plantations
Servian 75WG	Halosulfuronmethyl 750g/Kg	Control nut-sedge in maize and sugarcane, for control of sedges in small grains cereals
Solito 320EC	Pretilachlor + Pyribenzoxim	Control of weeds in rice under irrigation conditions
Terrier 240SL	Hexazinone	Pre-emergency weeds control in sugarcane
Terrier 750WG	Hexazinone	

Tornado 400SL Tornado 700WG	MCPA	Selective post emergence weeds control in sugar cane
Torpedo 480SC	Metribuzine	Pre-emergency weeds control in sugarcane
Twigazin 500 SL	Atrazin 500g/Lt	Pre-emergence weed control in maize and sugarcane
Volacet 900EC HE/0135	Acetochlor 900g/L	Control of weeds on sugarcane plantations
Volacet 900EC	Acetochlor 900g/L	
Volazinone	Hexazinone	
Volbuzine	Metribuzin 480g/L	
Volchlormuron	Chlorimuron-Ethyl	
Voliuron 800SC	Diuron 800g/l	
Volmet	Metolachlor	
Volmethalin 500EC	Pendimethalin 500g/l	
Volmetra 500SC	Atrazine 235g/L + Ametryn 245g/L	
Volmsma 720 SL	MSMA 720g/L	
Volsate 360	Glyphosate 360g/L	
Vulture 480SC	Triclopyr 480g/L	Selective weeds control in sugarcane.
Warrior 480EC	Clomazone 480g/l	
Wildbees 720 SL	2,4-D Amine 720g/l	Control of weeds on sugarcane plantations
1B: FUNGICIDES		
Tilt 250EC	Propiconazole 250g/L	Wheat, barley, sugarcane, coffee, grapevine against rust
Topcyclazole 75WP	Tricyclazole	Controlling foliar diseases in rice
Topsin-M 70% WP	Thiophanate-Methyl 70% w/w	Rice, wheat, tobacco, horticultural crops against blast leaf spot, powdery mildew, scabs and blight.
2. PROVISIONAL REGISTRATION CATEGORY (i.e. PESTICIDES REGISTERED FOR GENERAL USE FOR TWO YEARS)		
2A: INSECTICIDES		
Sapa Diazinon 60 EC	Diazinon	Coffee, tobacco, rice and sugarcane against chewing and sucking pests.
2B: HERBICIDES		
Basagran 480 g/l	Bentazone	Rice, maize, beans against broad leaved weeds and sedges.
Bass PL2	Bentazone+ Propanil	Rice, beans, maize against broad leaved weeds, sedges and grasses.
Buctril MC	Bromoxynil	Barley, maize, oats, wheat and rice against broadleaf weeds.
Diurex 80SC	Diuron	Sugarcane against weeds
Helmamine 720 EC	2, 4-D Amine	Cereals, sugarcane, sisal, coffee against post- emergence weeds
Sencor 480SC	Metribuzin	Sugarcane against weeds
Sindax 10WP	Bensulfuron + etasulfuron Methyl	On irrigated rice against broadleaf weeds and grasses
Stam UT-8 EC	Propanil + Phenothol	Rice against barnyard grass and cyperaceae
Stomp 500 EC	Pendimethalin	Sugarcane, cereals, cotton, sisal, rice against grasses and broad leaved weeds
Velpar 75DF	Hexazinone	Control of weeds in sugarcane
2C: NEMATICIDES		
Mocap 10G	Ethioprophos	Sugarcane, rice and maize against soil insects.
2D: RODENTICIDES		
Yasodion	Diphacinone	Against rats and mice in rice, sugarcane and maize
3. RESTRICTED REGISTRATION CATEGORY (i.e. PESTICIDES REGISTERED FOR RESTRICTED USE FOR TWO YEARS)		
3A: HERBICIDES		
Bamoxone 276SL	Paraquat 276g/L	Control of broad leaf and grass weeds in Sugarcane
Bramuron 536SC	Paraquat +Diuron	
Harpoon 200SL	Paraquat	Non selective weeds control in sugarcane
Harpoon ForteSL	Paraquat + Diuron	
Paraxone 200SL	Paraquat Dichloride	Weed control in sugarcane
Volcano paraquat SL	Paraquat	Weeds control in sugarcane plantations only
Volmuron	Paraquat + Diuron	For weed control in sugarcane
4. EXPERIMENTAL REGISTRATION CATEGORY (i.e. PESTICIDES REGISTERED FOR EXPERIMENTAL PURPOSES ONLY)		
4A: INSECTICIDE		

Regent 3GR	Fipronil	On rice against borers, gall midge and rice insects' pest.
4B: FUNGICIDES		
Luxan Carbendazim	Carbendazim	On sugarcane and other crops against fungal diseases
4C: HERBICIDE		
Argold 10% EC	Cinmethylin	Weed control in transplanted rice.
Cadre 24% SL	Imidazolinone	On sugarcane against Pre- and post- emergence weeds.
Calpen 500EC	Pendimethalin	On sugarcane for control of grass weeds and broadleaved weeds
Dinamic 700WDG	Amicarbozone	Various weeds on sugarcane
Ditex 50SC	Diuron	Pre-and post-emergency herbicide in various crops
Fer-Amine 720SL	2,4-D	Various grass and broad leaved weeds on rice against pre- and post-emergence
Focus ultra	Cycloxydim	On broad leaved crops to control annual and perennial grass weeds in rice
Garil	Trichorpyr	Weeds in cotton, onions, rice, sugarcane
Hamoxone 200SL	Paraquat	Weeds control in maize, grain, sorghum and sugarcane
Jumbo 480EC	Clomazone	Weeds in sugarcane ,tobacco, vegetables and rice
Merlin WG75	Isofluote	Pre-early post emergency herbicide in sugarcane
Nicanor 50WP	Metsulfuron-methyl	Cereals, pastures, rice and non -crop areas against weeds
Rainbow 25	Penoxsulam	Broad leaved weeds and grasses In rice fields
Ronstar 380 FLO	Oxadiazon	On broadleaved weeds and grasses in rice
Tancor 480SL	Metribuzin 480 SL	Controlling weeds in Sugarcane
Triclon	Triclopyr	Various weeds on sugarcane
Volazinone 750WDG	Hexazinone	Selective control of grasses and broadleaf weeds in ratoon sugarcane
Volcano MCPA 700 WSG	MCPA	Various weeds control in sugarcane
Volchlormuron 500 WDG	chlorimuron-ethyl	
Voliuron 800 WDG	Diuron	
Volmazone	Clomazone	
Volmetryne 750 WDG	Ametryne	
Volsate 500WSG	Glyphosate	

Appendix 7: Kilombero Valley: Soil reconnaissance and land suitability maps (Tanzania)



Sources: http://www.fao.org/geonetwork/srv/en/main.home
http://www.fao.org/geonetwork/srv/en/metadata.show?id=7057&currTab=distribution
http://eusoils.jrc.ec.europa.eu/library/maps/africa_atlas/

Appendix 8: Worldwide range concentration of heavy metals/metalloids in phosphatic, nitrogen, limestone fertilizers and animal farm-manures (mg/kg)

Metals	Phosphatic fertilizers	Nitrogen fertilizers	Lime fertilizers	Animal farm –manures
As	2-1,200	1-120	0.1-24	3-150
Ba	200	-	120-250	270
Cd	0.1-170	0.05-8.5	0.04-0.1	0.3-0.8
Co	1-12	5-12	0.4-3.0	0.3-24
Cr	66-600	3-19	10-15	5.2-55
Cu	1-300	1-15	2-125	2-60
Hg	0.01-1.2	0.03-3	0.05	0.09-26
Mn	40-2,000	-	40-1,300	-
Mo	0.1-60	1-7	0.1-15	0.05-3
Ni	7-38	7-38	10-20	7.8-30
Pb	7-225	2-1,450	20-1,250	6.6-350
Se	0.5-25	-	0.08-0.1	2.4
Sn	3-19	100-5,420	0.5-4	3.8
U	10-800	-	-	-
V	2-1,600	-	20	-
Zn	50-1,450	1-42	10-450	15-250

Source: Alloway (2012); Eckel *et al*. (2008); Kabata-Pendias and Mukherjee (2007)

Phosphatic fertilizers around the world can contain a wide range of most heavy metal(loid)s with the greatest variation being in Cd (1700-fold variation), V (800-fold) and As (600-fold). Relatively high Cd concentration in Phosphatic-fertilizers is a major cause of concern worldwide (Alloway, 2012).

Erklärung

"Ich versichere, dass ich die eingereichte Dissertation:

Applicability of a Biotest Battery Developed for Temperate Regions to Tropical Environments: Implications for Sustainable Wetland Management – A Case Study of Kilombero Ramsar Site

selbstständig und ohne unerlaubte Hilfsmittel verfasst haben. Anderer als der von mir angegebenen Hilfsmittel und Schriften habe ich mich nicht bedient. Alle wörtlich oder sinngemäß anderen Schriften entnommenen Stellen habe ich kenntlich gemacht.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Auch habe ich mich bisher noch mit keiner anderen Arbeit weder an dieser noch an anderen Universitäten einer Doktorprüfung unterzogen

Lüneburg, April 2015 Silvia F. Materu